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SOME METABOLITES OF CYATHUS HELENAE

BY

© HUBERT TAUBE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL, 1972

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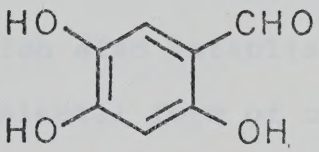
ABSTRACT

The metabolites of Cyathus helenae have been separated by chromatographic methods. The following compounds have been isolated from the crude extract called cyathin: chromocyathin (5), patulin (14), cyathin B_3 ($C_{20}H_{28}O_3$), cyathin C_5 ($C_{20}H_{26}O_5$), allocyathin A_4 ($C_{20}H_{30}O_4$), cyathin B_4 ($C_{20}H_{28}O_4$), cyathin A_3 ($C_{20}H_{30}O_3$), allocyathin B_3 ($C_{20}H_{28}O_3$), and cyathin A_4 ($C_{20}H_{30}O_4$). Patulin was identified by spectroscopic methods, chromocyathin was shown to be 2,4,5-trihydroxybenzaldehyde by spectroscopic methods and by synthesis.



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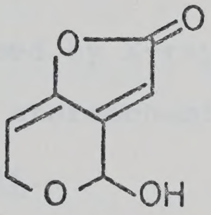

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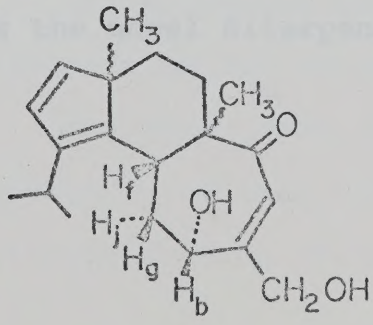
The structure of allocyathin B_3, which exists in solution in equilibrium between the tautomeric forms 40 and 41, was established by spectroscopic investigations of the parent compound and of its diacetyl and methyl ketal derivatives.



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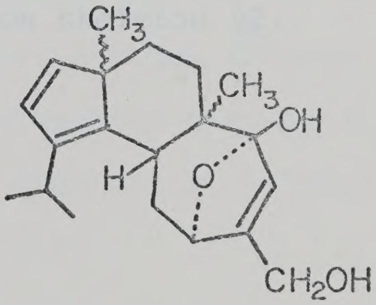

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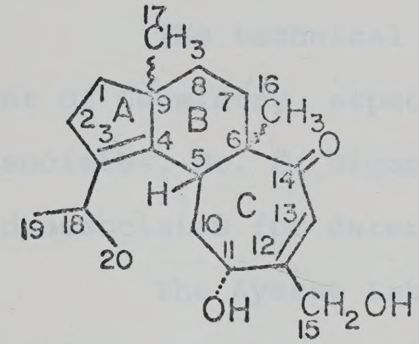
The spectroscopic data suggested that the keto form of cyathin A_3 has structure 44. This was confirmed by correlation of cyathin A_3 with allocyathin B_3 via the hydrogenation product 50.



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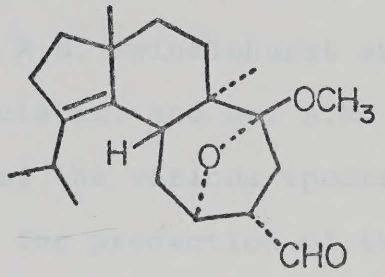

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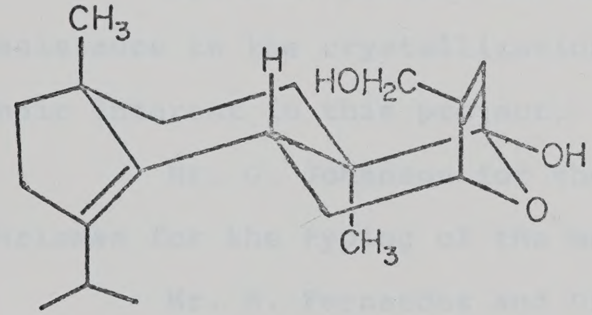
These assignments were confirmed by X-ray analysis which also established the relative stereochemistry of the hemiketal form of cyathin A_3 as in 46.



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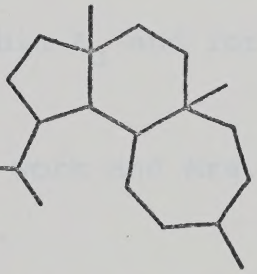

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It is believed that the other C_{20} compounds also possess the novel diterpenoid carbon skeleton 52.

ACKNOWLEDGEMENTS

The author wishes to thank:

The National Research Council of Canada and the University of Alberta for financial assistance.

The technical staff members of the Department of Chemistry, especially Mr. R.N. Swindlehurst and associates, Mr. G. Bigam and associates, and Dr. A.M. Hogg and associates for determination of the various spectra.

The Ayerst Laboratories for production of two cyathin preparations and biological testing.

Dr. A.D. Allbutt, Dr. B.N. Johri, Mr. M. Ouellet, and Mr. P. Yao for their cooperation in producing and separating cyathin.

Dr. M.J. Bennett and Dr. R.N. Tuggle for their assistance in the crystallization of cyathin A_3 and for their interest in this project.

Mr. G. Johanson for the graphic work and Mrs. C. Chrismas for the typing of the manuscript.

Mr. S. Fernandez and Dr. B. Strehlke for proofreading the manuscript.

Dr. M. Kodama for the analysis of the nmr spectra and assistance in the interpretation of other spectra.

His wife, Anna, for encouragement throughout the preparation of this thesis and substantial grammatical and stylistic editing of the manuscript.

Especially, Professor W.A. Ayer for his suggestions which initiated all advances made in this project and for the many helpful comments on the manuscript.

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I. INTRODUCTION

In 1965, Brodie discovered a new species of fungi growing in the Canadian Rocky Mountains[1]. He classified this fungus as belonging to the class Basidiomycetes, subclass Homobasidiomycetes, order Gasteromycetes, family Nidulariaceae (Bird's nest fungi), genus Cyathus, and gave it the name Cyathus helenae. This strain was found at an altitude of 7,000 ft near Mountain Park, Alberta, and was given the number 1,500 (Brodie Herbarium number). Subsequently C. helenae was also found at the following localities: Gillam, Manitoba; Drumheller, Alberta; the banks of the Miette River near Jasper, Alberta; and Elmore County, Idaho[2]. Only cultures of strain No. 1,500 were used throughout the work described in this thesis.

Olchowecki[3] succeeded in growing C. helenae in culture and obtained monosporous mycelia. He then investigated the mycelial characteristics and sexuality patterns of this culture and in the course of these investigations observed that a haploid culture of C. helenae No. 1,500 exhibited antagonism to a bacterial contaminant. Following up this accidental observation, he found that a substance (or substances) produced by C. helenae affected the growth of a number of unidentified as well as identified bacteria. Tests were then carried out with other

species of Cyathus: C. striatus, C. limbatus Tul. and C. poeppigii Tul. showed similar bacteristatic activity, whereas C. pallidus Berk. et Curt., C. bulleri Brodie, C. berkleyanus (Tul.) Lloyd and C. stercoreus (Schw.) De Toni did not. Olchowecki suggested the adoption of the name "cyathin" to refer to the antibiotic substance or substances produced by C. helenae, C. striatus and other species of Cyathus.

In 1952 Wilkins[4] had reported that C. striatus produces antibacterial substances. However, these were not further characterized.

Olchowecki carried out what might be considered the first chemical experiment of cyathin: autoclaving for 10 minutes at 120°. After this treatment it retained its biological activity, suggesting that its chemical constituents are heat stable.

Johri[5,6,7] continued to investigate the antibiotic activity of C. helenae and recorded an antimicrobial spectrum using a large number of microorganisms (actinomycetales, gram-positive and gram-negative bacteria and fungi, including "dermatophytes"). Cyathin was found to be active against the majority of these.

The method devised by Johri to isolate the antibiotic substance(s) from liquid cultures is still in use to date: the mycelium is removed by filtration and the broth is subsequently extracted with an equal volume of

ethyl acetate. The activity of cyathin is assayed by a paper-disc agar-plate method[8] using Staphylococcus aureus as the test organism. The diameter of the inhibition zone is indicative of the antibiotic activity of the material tested and thus can be quantitatively related to the concentration of the antibiotic in the particular solution.

At this point, it became desirable to characterize cyathin chemically. For this purpose large quantities of active substances were needed. These were obtained by growing the haploid strain No. 1,500-102 of C. helenae in a number of 500-, 1,000- or 2,800-ml Fernbach flasks (about 20 at a time), each being filled with 200, 400 or 1,000 ml of liquid medium, respectively. Originally a modified Brodie medium was used: dextrose, 20 g; yeast extract, 2.0 g; asparagine, 0.2 g; peptone, 0.2 g; glycerol, 6 ml; KH_2PO_4, 0.5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Fe_2(SO_4)$, trace; distilled water to make 1 liter. Later a chemically defined medium was substituted: dextrose, 30 g; asparagine, 1.5 g; KH_2PO_4, 1.0 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 2H_2O$, 0.25 g; thiamine hydrochloride, 0.15 g; distilled water to make 1 liter.

It was soon realized that the yields of cyathin both in terms of biological activity and weight of crude extract varied from batch to batch. Moreover, the composition of the extract was far from constant: some compon-

ents present in early extracts were not observed in later ones and new components were observed. The failure of the fungus to produce the desired material consistently has been attributed to changes in the chemical composition of the medium, and particularly to a phenomenon called "sectoring", i.e. micro- and macroscopic morphological variations of the mycelium, whose genetic basis has not been determined. This phenomenon had been previously observed by Olchowecki[2,3] on solid media.

Johri[5,6] undertook an exhaustive study of the chemical and physical factors influencing the growth and metabolism of the fungus, with the goal of optimizing and stabilizing the yield of the antibiotic complex. Static culture was compared with shake culture; the composition of the medium, the hydrogen ion concentration and the hours of light and darkness were changed systematically; the influence of various micronutrients, vitamins, carbon and nitrogen sources was evaluated. The tests were carried out in 125-ml Erlenmeyer flasks as culture vessels. To date, the knowledge gained from these investigations, however, has not been applied to large-scale production. It seems that with time the yields have become less reliable. An undetermined factor such as genetic stability may be responsible for the problem. It should also be mentioned that a number of different people have been involved in

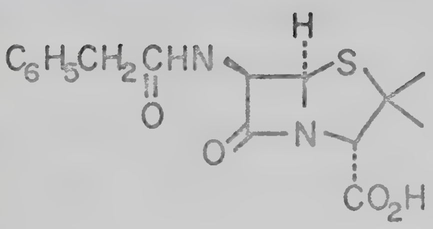
the production of the cyathin complex at different times: Dr. B. N. Johri of the Botany Department, University of Alberta; Dr. A. D. Allbutt, M. Ouellet and P. Yao of this Department; and Dr. C. Vezina of Ayerst Laboratories, Montreal. The changes in yield and composition might in part be due to this.

Because of technical problems and our lack of expertise in the area, it has not been possible as yet to produce large amounts of crude cyathin. Partly for this reason, our knowledge of the constituents of what has been called "crude cyathin", "cyathin complex" or "antibiotic cyathin" has advanced rather sporadically.

The purpose of the work reported in this thesis was to separate and characterize the active constituents of the cyathin complex and to determine their structures.

Four examples of metabolites isolated from fungal sources are listed in the Chart. Two of these are well-known antibiotics: penicillin G (1)[9], produced by *Penicillium notatum*, biogenetically derived from amino acids, and griseofulvin (2)[9], produced by *P. griseofulvum*, derived from acetate units. The antibiotic LL-Z1271α (3)[10], produced by an unidentified *Acrostalagamus* species, and diacetoxyscirpenol (4)[11], produced by *Fusarium sambucinum*, have terpenoid structures.

Chart



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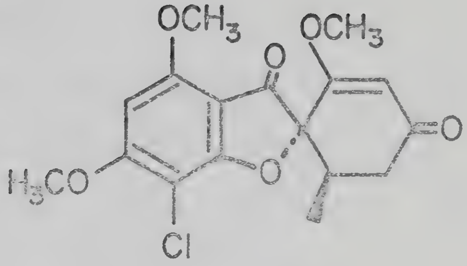

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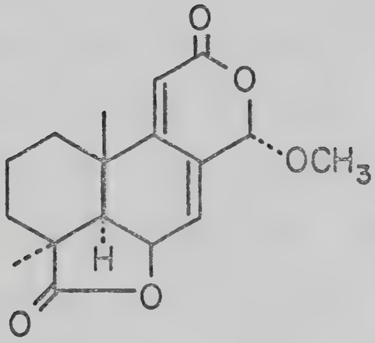

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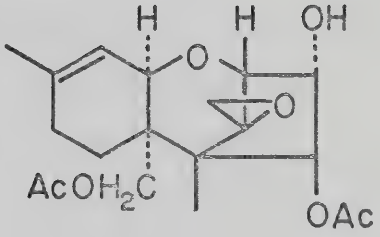

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It is apparent from this brief list that fungi produce a wide variety of metabolites. Therefore, we had to be prepared to encounter compounds belonging to any class of natural products when we first started to investigate cyathin.

II. RESULTS AND DISCUSSION

Characterization of Crude Cyathin

A dark-brown ethyl acetate solution of cyathin containing some undissolved material was received from the Botany Department in May 1968. After removal of the insoluble material by filtration, evaporation of the solvent gave material which we shall call "crude extract", weighing 3.68 g. Solubility tests with small samples led to the following results: the material was soluble in methanol, ethanol and acetone; partially soluble (as judged by the fact that the solvent became colored) in chloroform, methylene chloride and ether; insoluble in Skellysolve B (a mixture of saturated hydrocarbons, bp 65°C) and water. Considerable amounts of a sample were also soluble in dilute sodium hydroxide solution; however, it seemed that the material decomposed in alkaline solution since, with time, the solution took on a number of different colors.

"Partially purified material" was obtained by boiling the crude extract with a large amount of ether (0.58 g/600 ml ether) in the presence of charcoal, removing the insoluble fraction by filtration, and then removing the solvent by evaporation. Microanalysis of the residue gave the following results: C 63.9%; H 7.2%; N 0.5%; O 28.4% (by implication). The amount of nitrogen

appeared negligible; on the assumption that no elements other than carbon, hydrogen and oxygen are present, the following gross molecular formula results: $(C_{5.3}H_{7.2}O_{1.8})_x$. The low value for nitrogen excluded the possibility that the antibiotic substance is composed of alkaloids or peptides.

The "partially purified material" was subjected to routine spectral measurements. Both the ir and nmr spectra showed broad absorption bands, suggesting that the material was still impure. The ir, however, did show hydroxyl and carbonyl absorption (3200 - 3500, 1720 cm^{-1}).

The mass spectrum showed a base peak at m/e 154. The ultraviolet spectrum was very characteristic. In neutral ethanol there were absorption maxima at 241, 281 and 355 nm. These were shifted to 256 and 358 nm in dilute ethanolic sodium hydroxide. Such behavior is typical of phenols, a conclusion that was supported by the fact that the material gave a positive ferric chloride test.

Isolation and Structure of Chromocyathin

Although the substance which gave rise to the uv absorption was not necessarily the biologically active one, it was decided to first isolate this component, since it was easy (by uv) to locate it in individual fractions during the separation procedure. It was later found that

monitoring of the fractions by physical methods was in fact more useful than by biological methods, since a number of components in the extract gave positive biological tests. These were, therefore, not useful guides to purity.

The substance possessing the uv chromophore was called chromocyathin. Preliminary tests had indicated that it was a phenol, and it should thus be possible to purify it by ordinary acid-base separation.

Such treatment, however, gave rather poor results, probably due to the material's instability in alkaline solution. Pure chromocyathin could not be obtained in this way.

Separations of solids may be effected by selective removal of a component from a mixture by crystallization or precipitation. Such methods are at best only semi-systematic and were therefore considered unsatisfactory.

The more modern chromatographic techniques seemed more appealing since attempts at separation can be pursued more methodically. Gas chromatography was studied briefly but was soon abandoned because of the lack of volatility of the extract material.

Liquid-solid chromatography, both column (solvent descending) and thin-layer (solvent ascending), was explored next. Polyamide and alumina were not employed as adsorbents since the uv spectrum of a methanol solu-

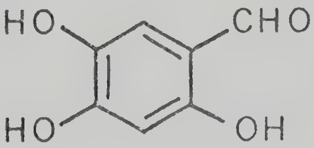
tion of the crude extract changed drastically on contact with these adsorbents. This indicated that chromocyathin decomposed or was so polar that, after contact with these adsorbents, it could not be eluted from them. When a solution of the crude extract was stirred with silica gel, the uv spectrum remained unchanged. This adsorbent therefore appeared well suited for chromatographic separations and has been used almost exclusively for all subsequent work connected with the cyathin problem.

The crude extract was subjected to column chromatography on silica gel or silicic acid (the latter being a silica preparation of smaller grain size than the former), and the fractions eluted were monitored by uv spectrometry. It was found that on silicic acid the solvent system chloroform-methanol (98:2) separated chromocyathin most selectively. However, by employing preparative thin-layer chromatography (ptlc), crystalline chromocyathin could be obtained more conveniently, as was shown by Dr. A. D. Allbutt of these laboratories.

This material was further purified by recrystallization from acetone-heptane. Chromocyathin proved to be rather insoluble in chloroform, and it was subsequently found that rather pure chromocyathin could be obtained by simple trituration of the total crude extract with chloroform.

The analytical sample was prepared by sublimation, followed by crystallization from methanol-chloroform, to yield yellow crystals, mp 216-219°, dec. The pure material had essentially the same uv spectrum as the crude extract: λ_{max} (methanol) 241,282,350 nm. The peak at m/e 154 in the mass spectrum of the crude extract proved to be the parent peak of chromocyathin. Exact mass determination of the peak at m/e 153 showed that this ion had the composition $C_7H_5O_4$; consequently, chromocyathin has the molecular formula $C_7H_6O_4$

The nmr spectrum, determined in hexadeuterioacetone, consisted of three sharp singlets at δ 6.42, 7.14 and 9.75, the ratio of the integrated areas being 1:1:1. The two former peaks are characteristic of aromatic and the latter of aldehydic protons. Since the aromatic protons show no coupling with each other, they must occupy para positions of a benzene ring. The remaining three protons appear sometimes as broad bands at δ 3.5 and 11.1; at other times they could not be detected. This was taken as an indication that they were present as hydroxyl protons. It follows that chromocyathin must be 2,4,5-trihydroxybenzaldehyde (5), a compound first described by Gattermann et al[12].



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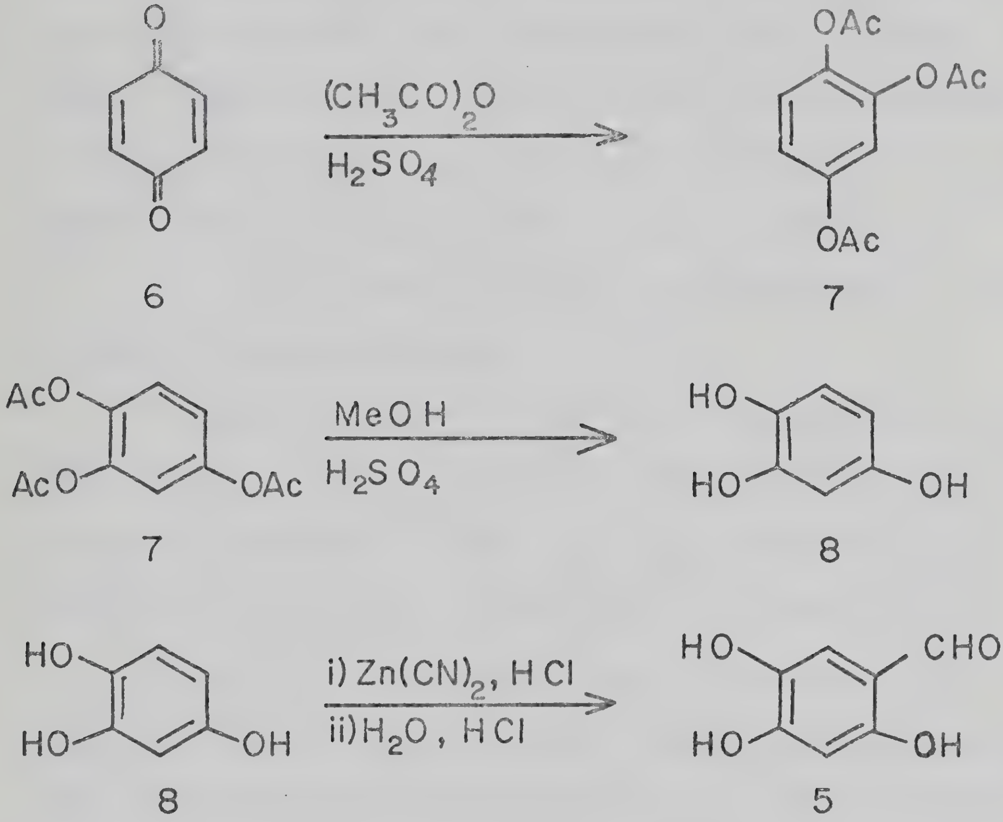
This assignment was confirmed by synthesis according to Scheme I, which was accomplished without difficulty since all reactions are well documented:

Scheme I



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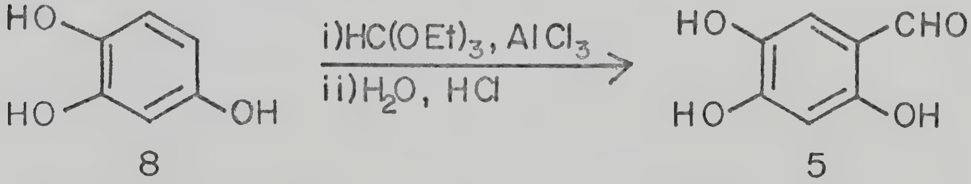
13, 14

12, 13

12, 15, 16

Use of the troublesome cyanide reagent was subsequently avoided by following a procedure published by H. Gross et al:



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Mass, ir, nmr and uv spectra of natural and

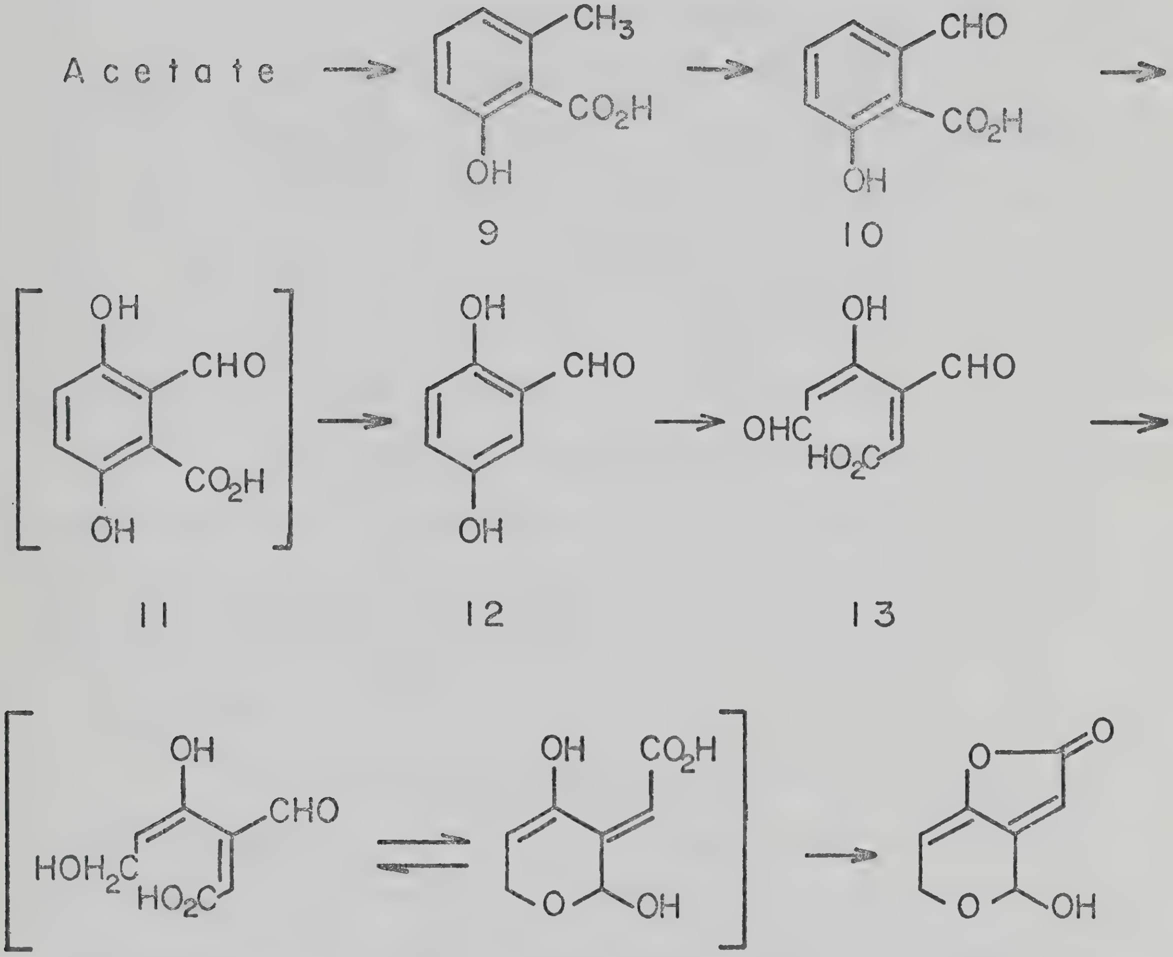
synthetic samples were indistinguishable. Both samples also had the same melting point 216-219° (reported 223°[12,17]); however, since melting was accompanied by decomposition, the melting behavior was not considered an absolutely reliable characteristic. 2,4,5-Triacetoxybenzaldehyde was therefore prepared as a derivative using standard procedures. Recrystallized samples of the natural and synthetic series both melted at 114-115° (reported 115°[18]). The melting point of mixed samples was not depressed.

Thus, the structure of chromocyathin is unequivocally established as **5**.

Both natural and synthetic **5** were found to possess some biological activity (Appendix 1). A proposed biogenetic pathway to patulin (**14**), a metabolite of *Penicillium patulum*, is outlined in Scheme II.[19]

It is conceivable that chromocyathin (**5**) constitutes a biogenetic intermediate between gentisaldehyde (**12**) and the ring-opened compound **13**. It is therefore tentatively suggested that chromocyathin is derived biosynthetically from acetate building blocks via intermediates **9**, **10**, **11** and **12**.

Scheme II


Acetate
CH3
CO2H
OH
9
CHO
CO2H
OH
10
OH
CHO
CO2H
OH
11
OH
CHO
OH
12
OH
CHO
OHC
HO2C
13
OH
CHO
HOH2C
HO2C
OH
CO2H
O
OH
13a
O
O
O
OH
14

Chromatographic Separation of the Cyathin Constituents

Johri[5,7] has shown by a method called bioautography that chromocyathin was not the only active principle of the crude extract. Consequently, we turned our attention to the chloroform-soluble portion of cyathin. Spectral examination indicated that this portion was heterogeneous; thin-layer chromatography on silica gel indicated that the mixture was indeed complex.

Liquid-solid chromatography was chosen to bring about separation of the complex mixture; we restricted ourselves to the use of silica gel (or silicic acid) as adsorbent; both thin-layer and column chromatography were employed.

Initially, both methods were used to effect a rough separation. Later on, it became apparent that column chromatography was more economical and less skill-demanding; it therefore became the preferred method to achieve preliminary separation of the complex into crude, partially purified fractions.

As mentioned earlier, the yield and composition of the cyathin extract varied from batch to batch. Approximately 20 such batches were produced during the time of my association with the problem. The details of the separation of a few of these cultivations are given in the Experimental section.

In order to obtain fractions consistent to at least some degree, they were grouped according to polarity (in the sense of chromatographic mobility). Cyathin B_3, chromocyathin, and cyathin A_4 (terminology described below) were constituents of the crude extract which did occur with a high degree of regularity and served as reference compounds. Ordinarily, cyathin B_3 was the least polar and cyathin A_4 the most polar (except for nonmoving material) component; chromocyathin is of intermediate polarity and is readily detected by its distinctive uv spectrum (as well as by other characteristics). Components with a polarity similar to cyathin B_3 are placed in group I, those with a polarity similar to chromocyathin in group III, and those with a polarity similar to Cyathin A_4 in group V. Components with intermediate polarities constitute groups II and IV. Solvent systems such as benzene-acetone-acetic acid (70:30:1) (B), chloroform-methanol (90:10) (I), and acetone-Skellysolve B (3:2) (N), when employed with silica gel tlc plates, were found suitable for separation of the cyathin components. These solvents were used to determine the polarities or R_f-values (distance travelled by test component/distance travelled by solvent front) of the cyathin components.

Column chromatography was carried out on silica gel (40 parts adsorbent:1 part cyathin). Chloroform

(containing the amount of ethanol present in the commercial product, 0.75%), chloroform-methanol (98:2) (G), and chloroform-methanol (95:5) (H) were used as eluents. The elution process was monitored by tlc and the individual fractions were assigned to one of the five polarity groups. Constituents of groups I and II were eluted by chloroform, those of groups III and IV by solvent system G, and those of group V by H.

Ptlc on silica gel G impregnated with an inorganic phosphor was employed as an alternative method to effect initial separation of the cyathin complex. Benzene-dioxane-acetic acid (100:25:1) (A) and benzene-acetone-acetic acid (75:25:1) (C) were used as developing media. Bands corresponding to the various constituents were detected under short-wave uv-light (254 nm). An example of both separation methods is given in the Detailed Experimental. As mentioned earlier, the crude extract is of variable composition, and, therefore, the separation results differ from batch to batch.

Fractions obtained from an initial separation often constituted mixtures (groups II, III and IV) or were at least still impure (groups I and V). Ptlc was employed, sometimes repeatedly, to achieve further purification. A fraction was considered chromatographically pure if it gave rise to only one spot on tlc. It should

be noted that chromatographic purity is not identical with absolute purity; material pure according to chromatography might still be heterogeneous by other criteria.

Chromatographically pure substances were subjected to high resolution mass spectrometry (hrms). Their molecular formulas were found by determining the composition of the ion which gave rise to what appeared to be the parent peak. No attempt was made to confirm molecular formulas by microanalysis, since it was uncertain whether the samples would meet the stringent requirements of purity, and since the amounts of sample available were often limited.

Most of the components of cyathin turned out to be of the composition $C_{20}H_xO_y$. The following system of trivial nomenclature has been adopted. Compounds where $x = 30$ are called cyathin A, where $x = 28$, cyathin B, and where $x = 26$, cyathin C. The value of y appears as a subscript after the capital letter; e.g. cyathin C_5 is $C_{20}H_{26}O_5$. Isomers of the first isolated cyathins are placed in the allo series; e.g. both cyathin A_4 and allocyathin A_4 have the molecular formula $C_{20}H_{30}O_4$.

Table I lists the constituents which have been isolated from the crude cyathin extract.

The mass and ir spectra of cyathin B_3, allocyathin A_4, cyathin C_5, cyathin B_4 and cyathin A_4 are

reproduced in Figures 1-10. Spectra of cyathin C_5 and cyathin A_4 were provided by the courtesy of Dr. A. D. Allbutt. It should be understood that the samples were not necessarily chemically pure. However, the spectra might be useful for future reference. Cyathin A_3 is not included here, since it will be discussed in more detail below.

Patulin (14), recognized by its mass, ir, nmr and uv spectra, was isolated from two batches grown by the Ayerst Laboratories. It is possible that its occurrence may be due to contamination of the fungal culture, although this remains to be proven. The fact that chromocyathin may be an intermediate in the biosynthesis of patulin opens the possibility that the growth conditions used by the Ayerst workers were sufficiently different to cause transformation of chromocyathin into patulin. It should also be noted that no chromocyathin was isolated from the two Ayerst preparations.

Table I. Chromatographic Data of Cyathin Constituents (Silica Gel G)

	R_f in Solvent System					
Group	A	B	C	H	I	Constituent
I	-	0.56	-	0.6	-	cyathin B_3
II	0.55	0.54	0.54	-	-	cyathin C_5
	-	0.5	-	0.35	-	allocyathin A_4
III	0.44	0.51	0.51	-	-	chromocyathin
	-	-	-	0.25	0.45	patulin
	-	0.39	0.50	-	-	cyathin B_4
	-	0.31	0.36	0.2	0.4	cyathin A_3
	-	0.31	0.36	0.2	0.4	allocyathin B_3
IV	-	-	-	-	-	unidentified
V	-	0.09	0.06	-	-	cyathin A_4

See Experimental for abbreviations of solvent systems.

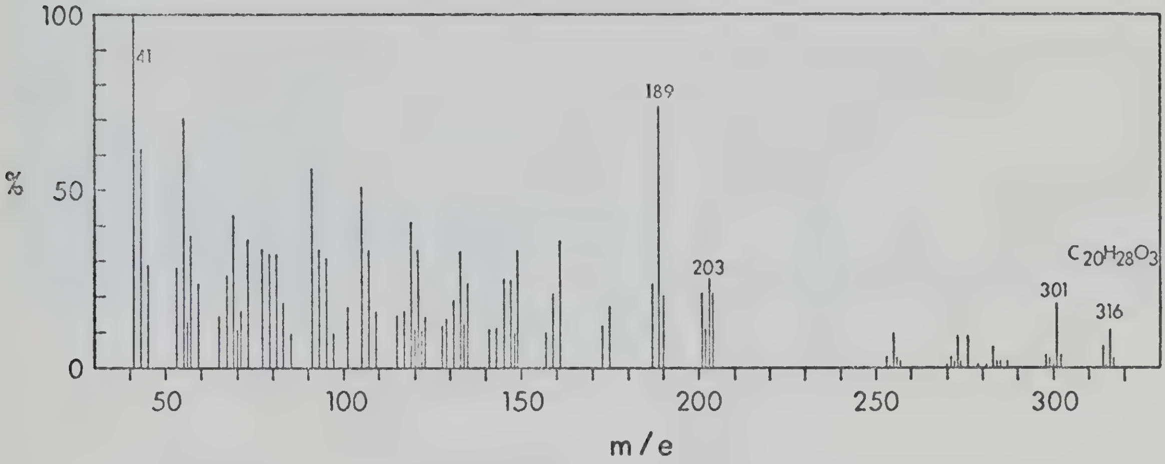


Figure 1. Mass spectrum of cyathin B_3

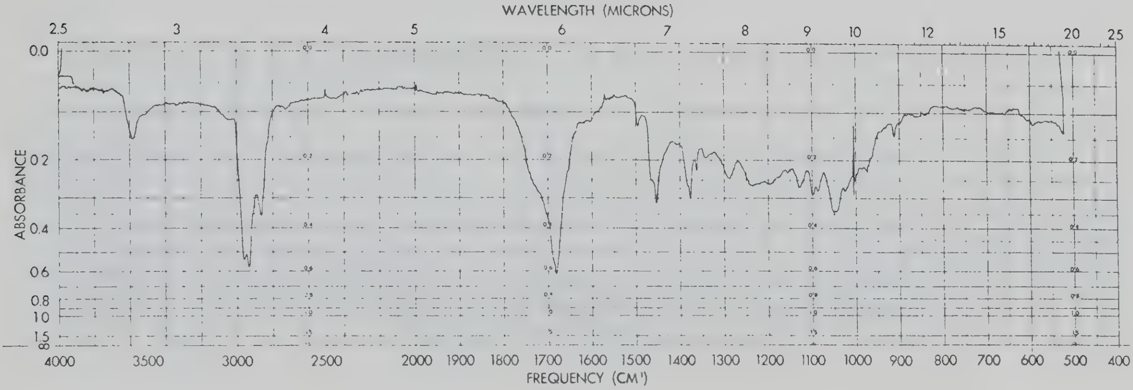


Figure 2. Ir spectrum of cyathin B_3 ($CHCl_3$)

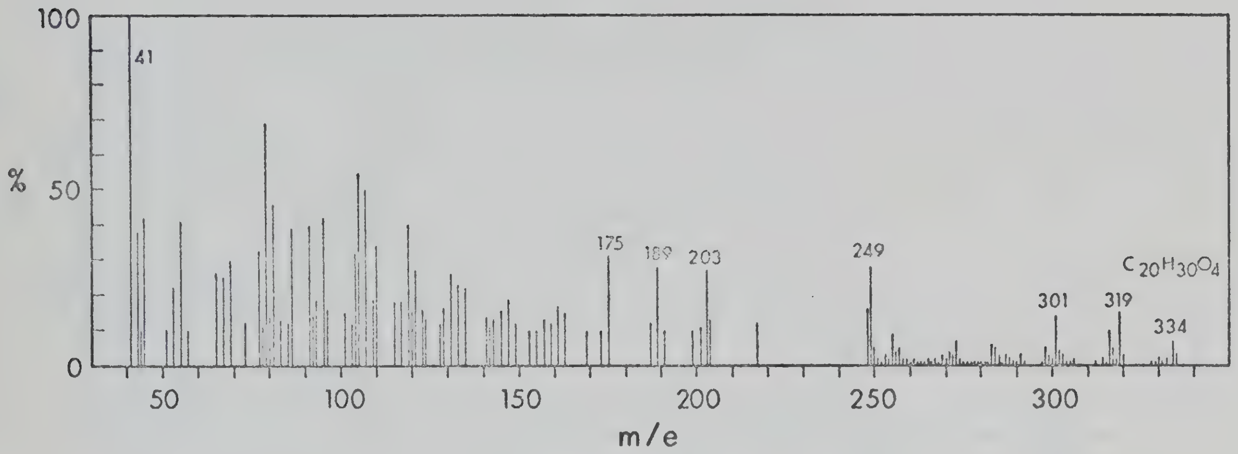


Figure 3. Mass spectrum of allocyathin A_4

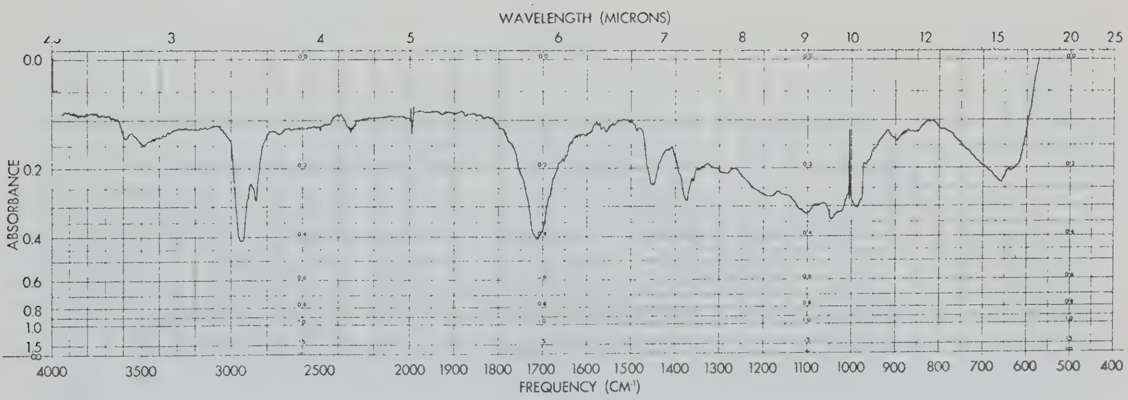


Figure 4. Ir spectrum of allocyathin A_4 ($CHCl_3$)

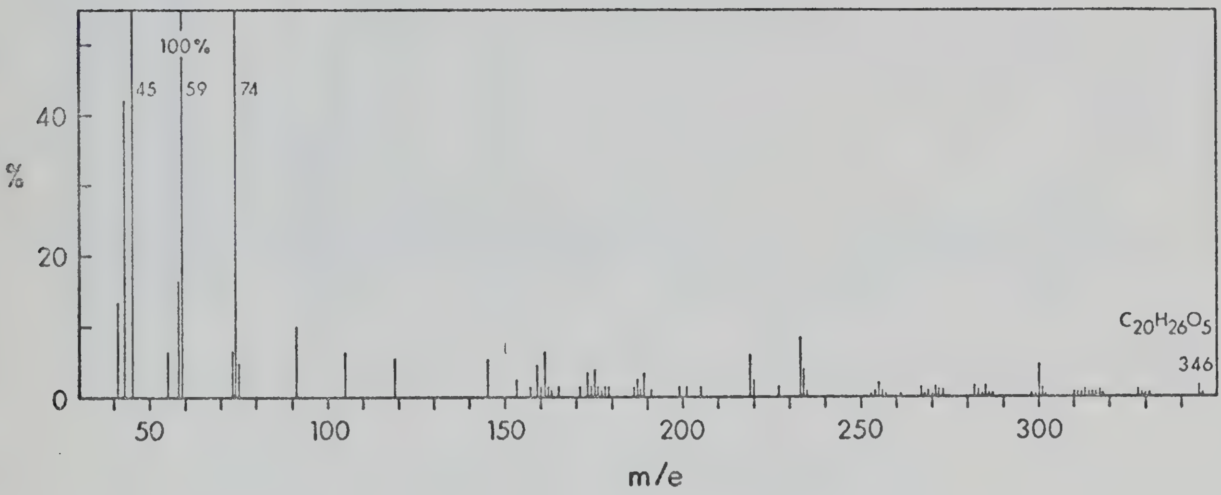


Figure 5. Mass spectrum of cyathin C_5

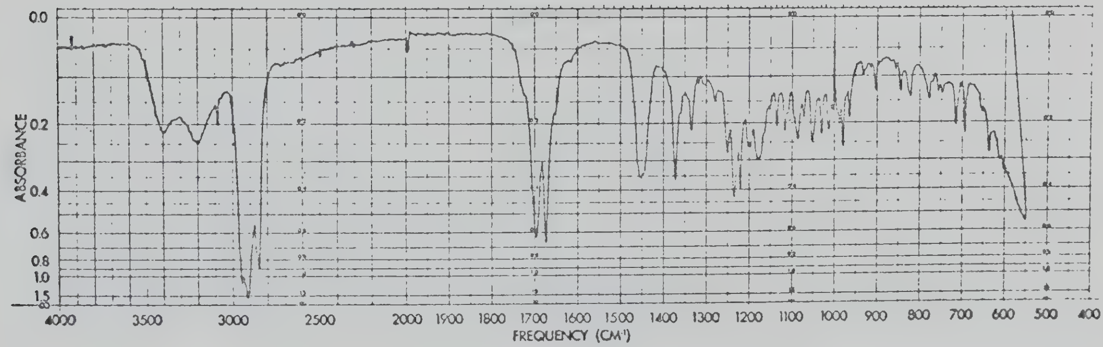


Figure 6. Ir spectrum of cyathin C_5 (Nujol)

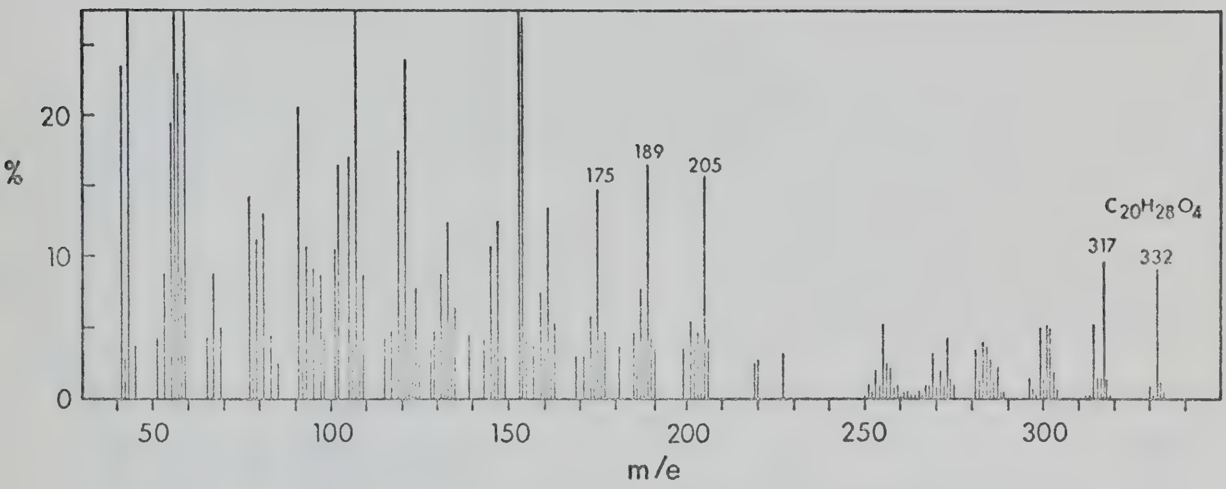


Figure 7. Mass spectrum of cyathin B_4
Peaks at 43 (51), 56 (100), 59 (64), and 153 (28) are off-scale.

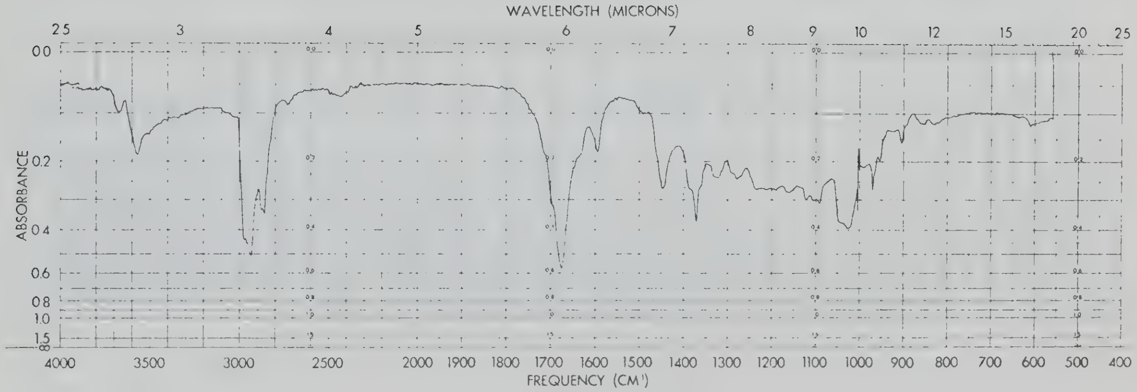


Figure 8. Ir spectrum of cyathin B_4 ($CHCl_3$)

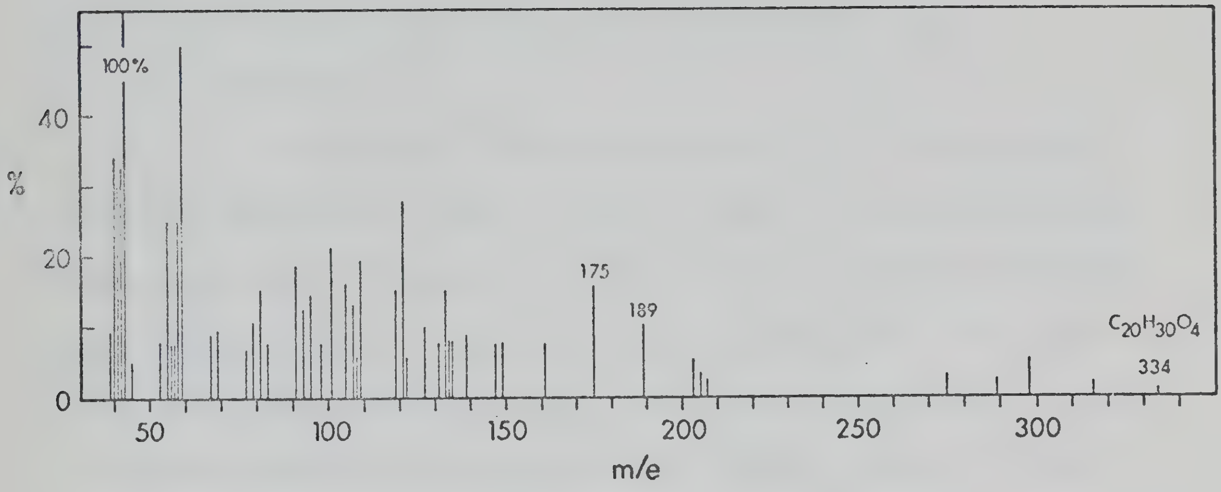


Figure 9. Mass spectrum of cyathin A_4

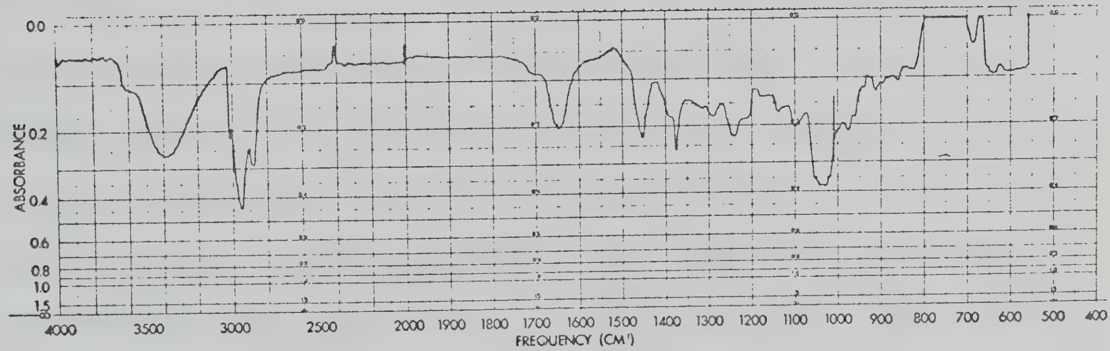


Figure 10. Ir spectrum of cyathin A_4 ($CHCl_3$)

The Structure of Cyathin A_3 and Allocyathin B_3

1) "A_3-Mixture"

A substance similar in polarity to chromocyathin was detected in most cultivations of Cyathus helenae. When fraction III (group III) from the crude chromatographic separation was subjected to preparative thin-layer chromatography on silica gel, this substance was obtained in chromatographically pure form (single spot on silica gel tlc). The mass spectrum had the parent peak at m/e 318, the exact mass corresponding to the composition $C_{20}H_{30}O_3$. According to the convention outlined earlier, the substance was called "cyathin A_3".

Reactions such as acetylation, catalytic hydrogenation, and sodium borohydride reduction were carried out with "cyathin A_3", resulting in the formation of the corresponding acetylated, hydrogenated, and reduced products. As was the case with "cyathin A_3" itself, none of the derivatives were crystalline except for small amounts of one of the hydrogenolysis products obtained from acetylated "cyathin A_3". The absorption bands in the ir spectra always appeared rather broad, and the mass spectra showed many peaks which were difficult to interpret (e.g., peak at M^+-2). The nmr spectra could be analysed to some degree but usually showed some anomalies.

Also, the spectra were not always reproducible; in one particular case, the mass spectrum of the diacetyl derivative of "cyathin A_3" failed to show the previously observed parent peak at m/e 402 (318 + 2 x 42), but gave an apparent parent peak at 400.

These facts suggested that "cyathin A_3" was not homogeneous.

When the nmr spectrum of the diacetyl derivative was carefully reexamined, it was found that a complete analysis of those signals which are well defined was possible, if it was assumed that "cyathin A_3" was a mixture of two compounds: compound A and its dehydro derivative, compound B. The 220 MHz nmr spectrum is reproduced in Figures 11 and 12. Signals attributed to compound A are analysed in the lower row; those attributed to B, in the upper one. In this particular instance, the ratio of amounts of compound A : compound B is approximately 1 : 1, as estimated by the integration of the peaks. More often, it was in the range of 2:1 to 4:1, the compound giving rise to the two additional signals y and z being the minor component.

The mixture then became known as the "A_3-mixture", consisting of a major component A_3 and a minor component A_3'.

When analytical tlc experiments with "A_3-mixture"

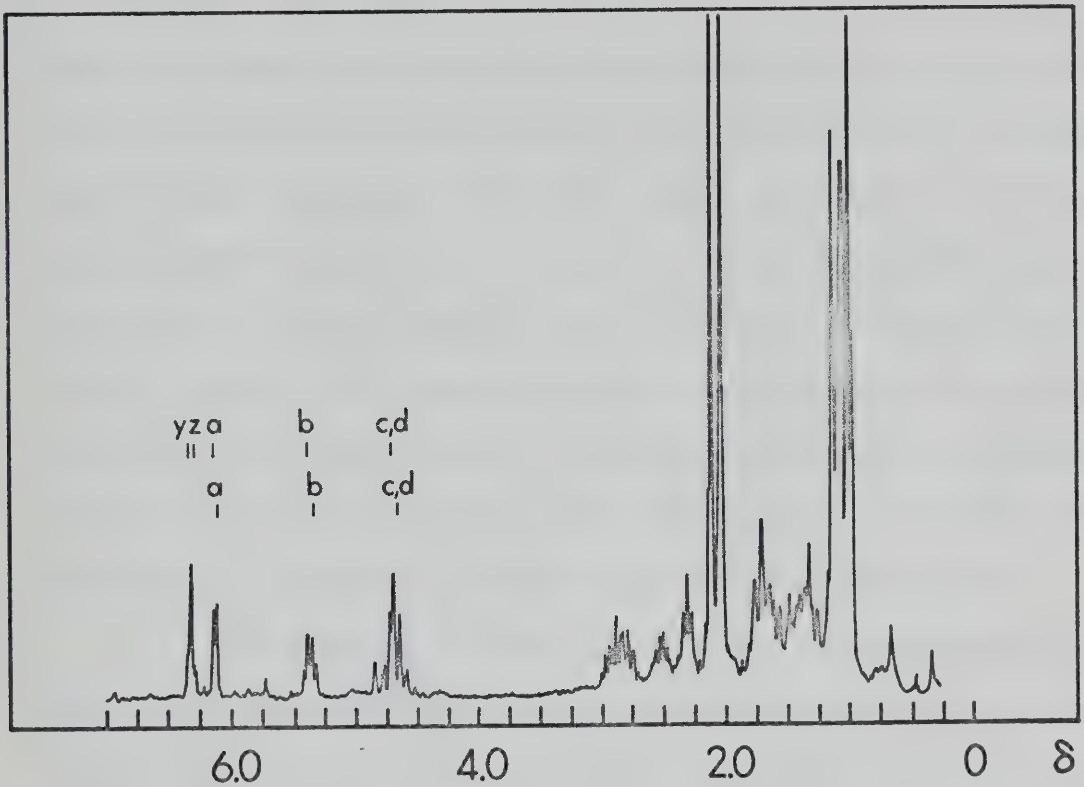


Figure 11. Spectrum of the diacetyl derivative of the "A_3-mixture" ($CDCl_3$)

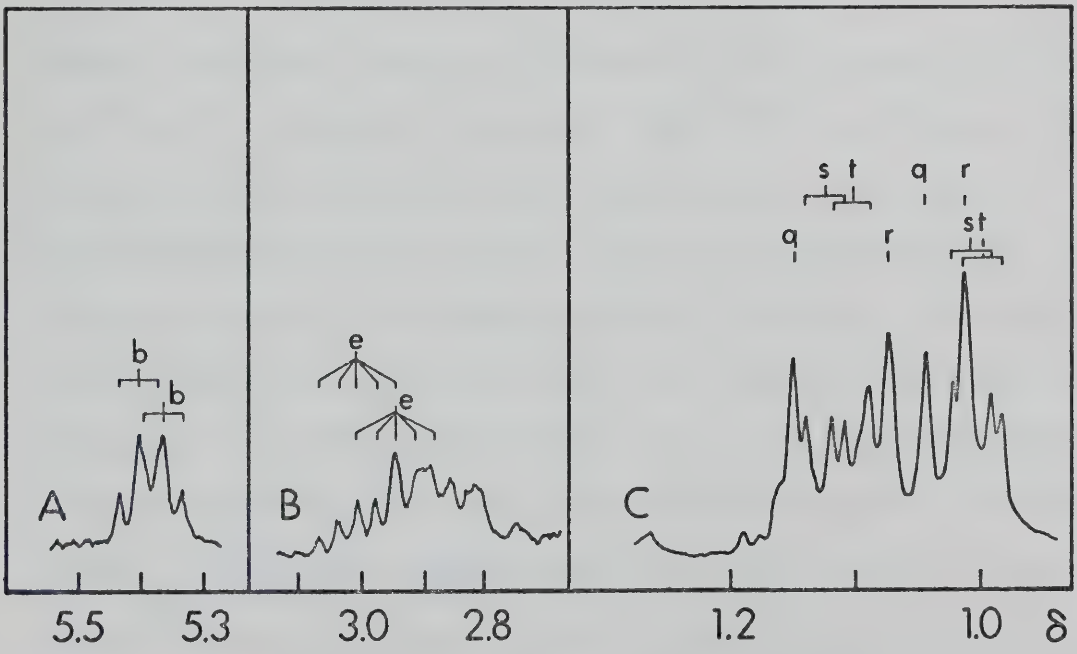


Figure 12. Portions of the expanded 220 MHz nmr spectrum of the diacetyl derivative of the "A_3-mixture"

were repeated, it was found that two spots of different color could be observed on the chromatogram if the detection method (spraying with 30% H_2SO_4 followed by heating) was followed carefully. (If not observed immediately, the original two spots merged into one black, somewhat enlarged spot.) These overlapped so much that a useful separation could not be accomplished. The more elaborate techniques of multiple or two-dimensional development, as described by Stahl[20], did not lead to appreciable improvement.

In natural product chemistry, chromatography on silica gel impregnated with silver nitrate has been used to separate compounds with isomeric double bonds[21]. Compounds in which the double bond is more exposed (monosubstituted > disubstituted > trisubstituted > tetrasubstituted) are more strongly adsorbed by the argentated silica gel due to π-complex formation; the differing degree of adsorption is exploited for separation. Similarly, an unsaturated compound is more strongly adsorbed than a saturated compound of the same series. Reference 22 gives an example from the steroid field. In lipid chemistry, chromatography on argentated silica gel is a standard method for separating fatty acids of differing degrees of unsaturation, or cis and trans isomers of fatty acids. Both column[23] and thin-layer chromatography[24] have been used.

These reports led us to anticipate that a much

improved separation of the "A_3-mixture" would result from chromatography on argentated silica gel. Consequently, the effectiveness of analytical tlc on silica gel impregnated with 1%, 2%, 5%, 10% and 25% silver nitrate was systematically investigated. Separation of the two compounds of the "A_3-mixture" was improved in this order; however, the spots resulting from chromatography with silica gel impregnated with 25% silver nitrate were ill defined. Silica gel impregnated with 10% silver nitrate appeared to be the adsorbent which gave optimal results and has been used for all subsequent chromatographic work, both for analytical and preparative purposes, using either column or thin-layer chromatography.

The influence of a number of solvent systems on the quality of separation was also investigated. A chart published by Neher[25] was very helpful in correlating the polarities of different solvent systems. Acetone - Skellysolve B (3:2) (solvent system G) appeared superior to systems such as ethyl acetate - Skellysolve B (3:2), or acetone - ether (3:7), when used in the tlc work.

Keeping in mind that theory predicts that the less polar component would be the more saturated one, it can be concluded that the component corresponding to the spot with the higher R_f-value should be the major component A_3 , whereas the minor component A_3' should have the

lower R_f-value.

Under optimal conditions, the R_f-value of the major component A_3 was 0.60; that of the minor component A_3', 0.51. Such a difference in the R_f-value should be sufficient to allow easy preparative separation by both thin-layer and column chromatography.

For analytical purposes, the same detection method (spraying with 30% H_2SO_4) as for ordinary tlc was used. The component with the higher R_f-value takes longer to develop a color. At first the spot is rosé-red, then gradually turns black. The slower-moving component stains much more rapidly and its color is much deeper, a dark purple-blue. The difference in intensity gives the impression that the more polar material is the major component. However, the fact that it chars more rapidly must be due to the additional unsaturation.

Initially, no report of a nondestructive detection method in connection with tlc on argentated silica gel could be found in the literature. Separation by column chromatography was attempted. Using Skellysolve B - acetone (3:1) (M) as a developing solvent, the R_f-value of the two components was of the order of 0.05. This solvent should have the right elutropic power to effect separation.

When the experiment was actually carried out, it was found that solvent system M was still too polar and

no separation was achieved; when Skellysolve B - acetone 7:1 (K) was used instead, a number of fractions was obtained containing the major component in excellent purity, as evidenced by tlc. To our delight, one of the fractions crystallized spontaneously when left unattended overnight. However, the minor component could not be obtained in such high purity, although the later fractions were highly enriched in this material.

Repeated application of this method could probably have achieved separation of the minor component in high purity. However, the process would have been laborious. It was also anticipated that each purification step would result in some losses and that eventually the desired component could have been isolated, but in very poor yield.

The literature was again searched for nondestructive detection methods in connection with argentation chromatography. One article[22] reported the use of a 10% aqueous solution of potassium bromide as a spray reagent. Supposedly, the adsorbent layer becomes dark grey (due to formation of elemental silver) on which the zones containing organic material stand out as white areas. However, when this method was used, no dark gray background was formed and no spots could be detected.

Further literature search[26,27,28] uncovered a

method which proved successful: when argentated silica gel layers are sprayed with a 0.2% solution of 2',7'-dichlorofluorescein (or 2',7'-dibromofluorescein) in ethanol, a pink coloration of the layers occurs. This is due to complex formation of the silver ions with 2',7'-dichlorofluorescein. If organic material is present in sufficient quantity, it is detected in the form of greenish spots (zones) in ordinary daylight. The sensitivity of the method is increased by observing the plates under a uv lamp (325 nm). Organic compounds then appear as light spots (zones) on a dark background. Ordinarily, the quantities separated were so small that detection could only be achieved using the uv lamp.

Chromatography of the "A_3-mixture" under these conditions led to a good separation. The components were eluted from the adsorbent with ether. Initially, acetone was used for this purpose, but proved to be unsatisfactory since this solvent also eluted 2',7'-dichlorofluorescein and traces of silver salts. The components obtained in this way proved to be homogeneous on analytical tlc (silver nitrate-silica gel). The major component was shown by hrms to possess the molecular formula $C_{20}H_{30}O_3$ and is called cyathin A_3. The minor component has the molecular formula $C_{20}H_{28}O_3$ and, since it was the second component of this composition isolated from crude cyathin, it is named allocyathin B_3.

2) Cyathin A_3

Cyathin A_3 was further purified by crystallization. If the separation procedure outlined above was followed, crystals of cyathin A_3 were obtained consistently. Acetone and ether may be used for recrystallization although cyathin A_3 is fairly soluble in these solvents and the recovery is low. Cyathin A_3 is much less soluble in benzene and particularly benzene-cyclohexane mixtures. These systems were found more suitable and were employed to obtain large single crystals. Single crystals of approximate size 0.1 x 0.1 x 0.1 mm, at another time 1.0 x 0.3 x 0.3 mm, were deposited from these solvents. These crystals were suitable for X-ray crystallographic studies.

By determining the density and the dimensions of the unit cells of these crystals, the molecular weight of cyathin A_3 was independently calculated. A mixture of cyclohexane ($d = 0.78\ g/cm^3$) and carbon tetrachloride ($d = 1.59\ g/cm^3$) was adjusted so that the buoyancy of an added crystal was neutral. The density of the mixture was measured in a pycnometer and found to be $d = 1.157\ g/cm^3$ (±0.002).

The crystallographic studies revealed that the cyathin A_3 crystals belonged to the orthorombic system, space group $P2_12_12_1$. The unit cell had the dimensions

a = 18.228, b = 15.105, c = 6.629 Å (volume of unit cell = 1825.1 $Å^3$).

The molecular weight (mw) was calculated according to the formula:

mw = d x volume of unit cell x Avogadro number/number of molecules per unit cell

Assuming four molecules per unit cell, the molecular weight calculated in this way is 317.85, confirming the mass spectrometric measurements.

The mass spectrum of cyathin A_3 (Figure 13) has an intense characteristic peak at m/e 191. The composition of the corresponding ion is $C_{14}H_{23}$. This allows the tentative conclusion that the cyathin A_3 molecule is composed of two portions: a large $C_{14}H_{23}$ unit containing no oxygen atoms, and a small C_6 fragment carrying all three oxygen atoms. However, it should be kept in mind that hydrogen migration can occur, and, more seriously, that the large fragment could have lost small fragments (such as H_2O) during fragmentation, making it unsure that the three oxygens are on the six-carbon unit.

Peaks at m/e 303 (M - CH_3), 300 (M - H_2O), and 275 (M - C_3H_7) indicated the presence of hydroxyl, methyl and propyl groups in the cyathin A_3 molecule.

The ir spectrum of a chloroform solution of cyathin A_3 (Figure 14) displayed broad absorption bands.

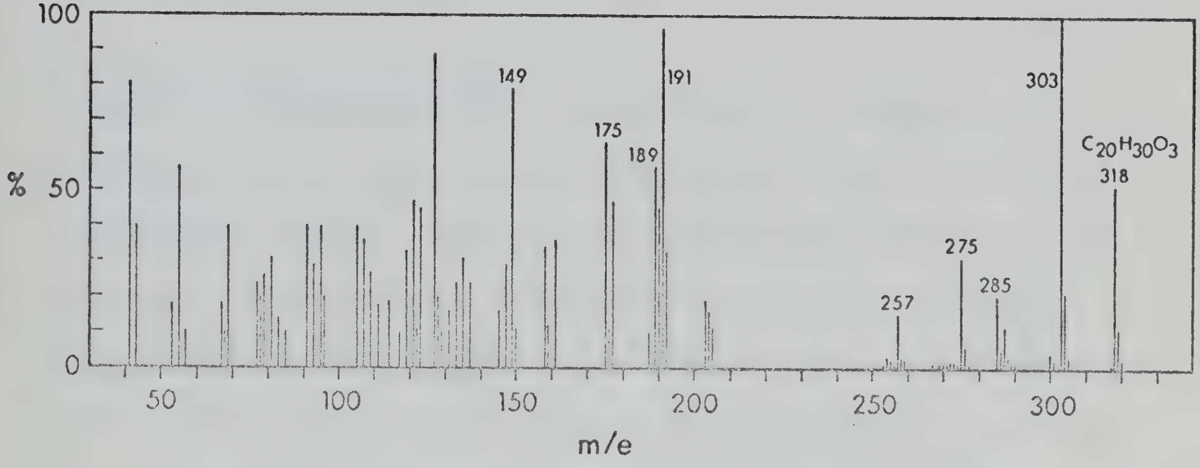


Figure 13. Mass spectrum of cyathin A_3

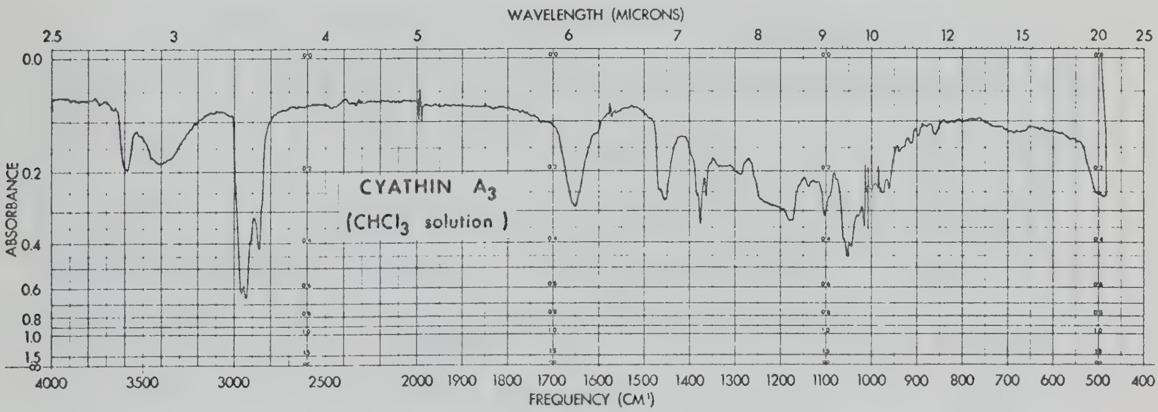


Figure 14. Ir spectrum of cyathin A_3 ($CHCl_3$)

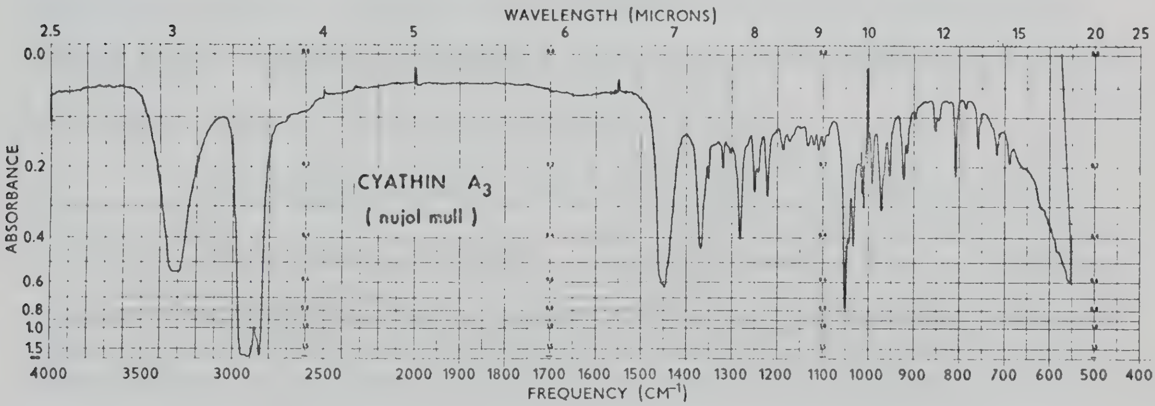


Figure 15. Ir spectrum of cyathin A_3 (nujol)

This was unexpected since the purity of the sample was assured and, as a rule, pure samples give rise to sharp absorption bands. Absorptions at 3600 and 3400 cm^{-1} are assigned to free and hydrogen-bonded hydroxyl groups. The broad band of medium intensity at 1650 cm^{-1} remained unexplained. C,C-Double bonds, particularly in the case of enol ethers, and carbonyl groups of an α,β-unsaturated ketone were considered as possibilities.

Solid-phase ir spectra of cyathin A_3 were obtained by employing both the KBr-disc (not shown) and the nujol-mull techniques (Figure 15). The nujol spectrum shows no absorption in the region 1600-1700 cm^{-1}; the KBr spectrum shows a broad absorption at 1650 cm^{-1}. At the time, this absorption was attributed to cyathin A_3, but now it is clear that it is due to water present in the KBr-disc.

A Raman spectrum, obtained on a sample of microcrystalline cyathin A_3, showed a rather sharp band at 1653 cm^{-1} with a shoulder at 1658 cm^{-1}, indicating the presence of two C,C-double bonds.

Hindsight allows the following interpretation of the ir and Raman spectra: in the solid state, cyathin A_3 has a masked carbonyl group which is partially unmasked in solution. The position of the carbonyl absorption, below 1700 cm^{-1}, points to an α,β-unsaturated carbonyl

group. The unresolved absorption bands of the Raman spectrum are attributed to two C,C-double bonds. At the time that the spectra were first determined, however, it was not possible to reach these conclusions and the spectra caused considerable confusion.

The nmr spectrum (Figure 16) of cyathin A_3, determined in deuteriochloroform solution, again showed rather broad absorptions, unexpected for a pure substance. Phenomena such as conformational mobility, exchange of hydroxyl protons and tautomeric equilibration may be responsible for these broad absorption bands. As the nmr spectra of cyathin A_3 derivatives lend themselves more readily to interpretation, the spectra of the parent compound will not be discussed further.

Uv spectra of cyathin A_3 (Figure 17) were recorded in both methanol and dioxane. No distinct absorption maximum is detectable below 250 nm in either solvent, although there is an inflection at 226 nm in methanol ($\varepsilon = 2,500$), suggesting some type of conjugated system.

There is a definite absorption maximum above 300 nm: in methanol solution it appears at 315 nm with $\varepsilon = 51$. It should be noted, however, that the maximum is not well resolved from the absorption mentioned above, which is presumably due to the $\pi - \pi^*$ transition. In order to obtain the true λ_{max} and ε-values for the transition

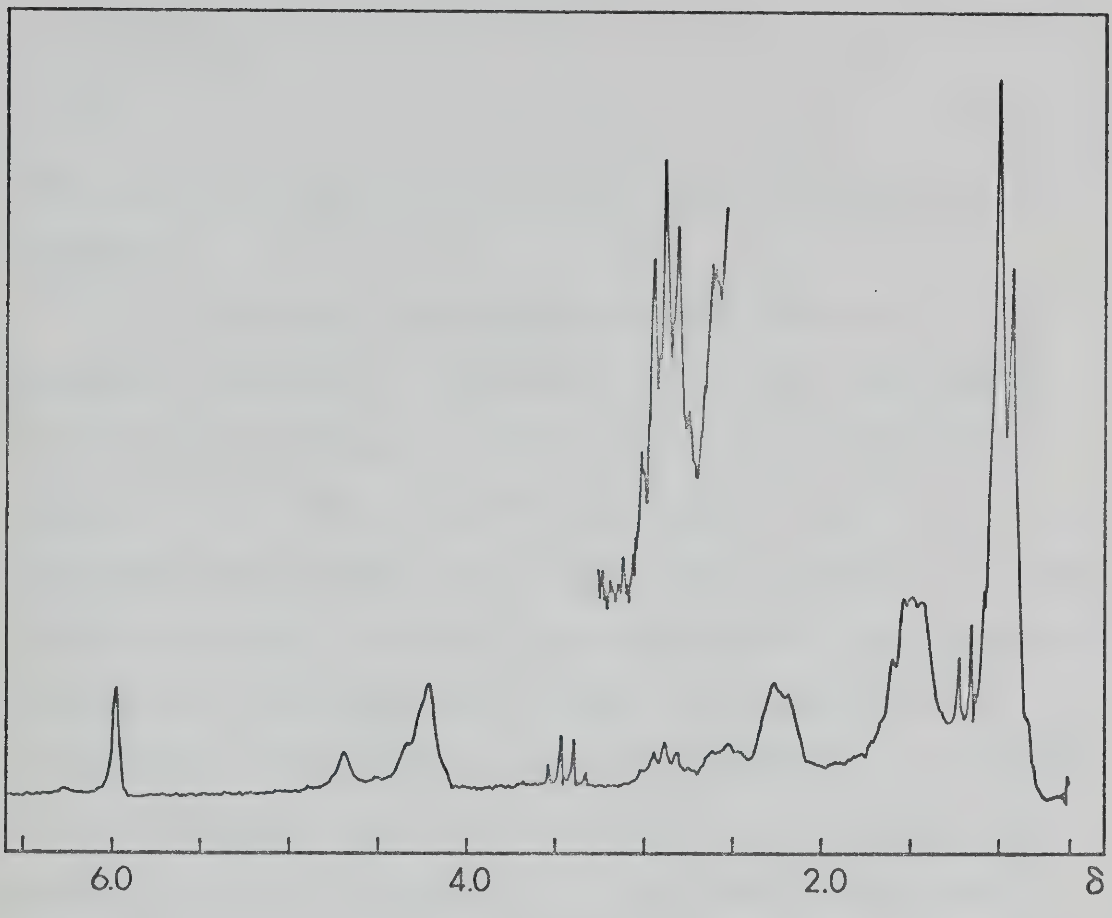


Figure 16. 100 MHz nmr spectrum of cyathin A_3 ($CDCl_3$); impurities at δ 3.4 and 1.1

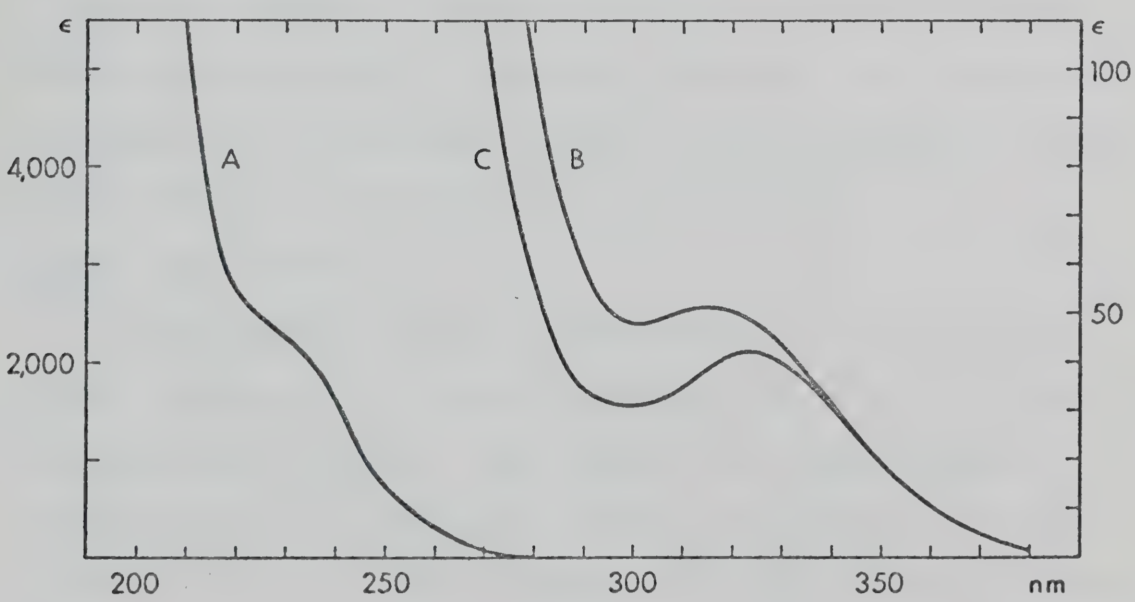


Figure 17. Uv spectrum of cyathin A_3; A, B in methanol; C in dioxane; A left scale; B, C right scale

under consideration, the apparent values would have to be corrected.

In dioxane, the absorption bands due to the two different transitions are better resolved. The maximum of the long-wavelength band is found at 324 nm with ε = 42; due to the better resolution, these values should be considered more significant than those in methanol. Both the position and extinction coefficient of this absorption maximum are characteristic of the n-π* transition of an α,β-unsaturated carbonyl group of an aldehyde or ketone[29].

The difference of the uv spectra in dioxane and methanol is easily explained. It is well established[30] that in polar solvents such as methanol, π-π* transitions are shifted to higher, whereas n-π* transitions are shifted to lower wavelengths. This results in diminished resolution of the two bands. Non-protic solvents such as dioxane give rise to improved resolution and, therefore, should preferentially be used for uv spectroscopy of α,β-unsaturated ketones.

The presence of an α,β-unsaturated ketone is also evident from optical rotary dispersion (ord) and circular dichroism (cd) studies. The spectra were again measured in both methanol and dioxane (Figures 18 and 19). Cyathin A_3 gives rise to a plain negative ord curve on which there is superimposed a positive Cotton effect centered at

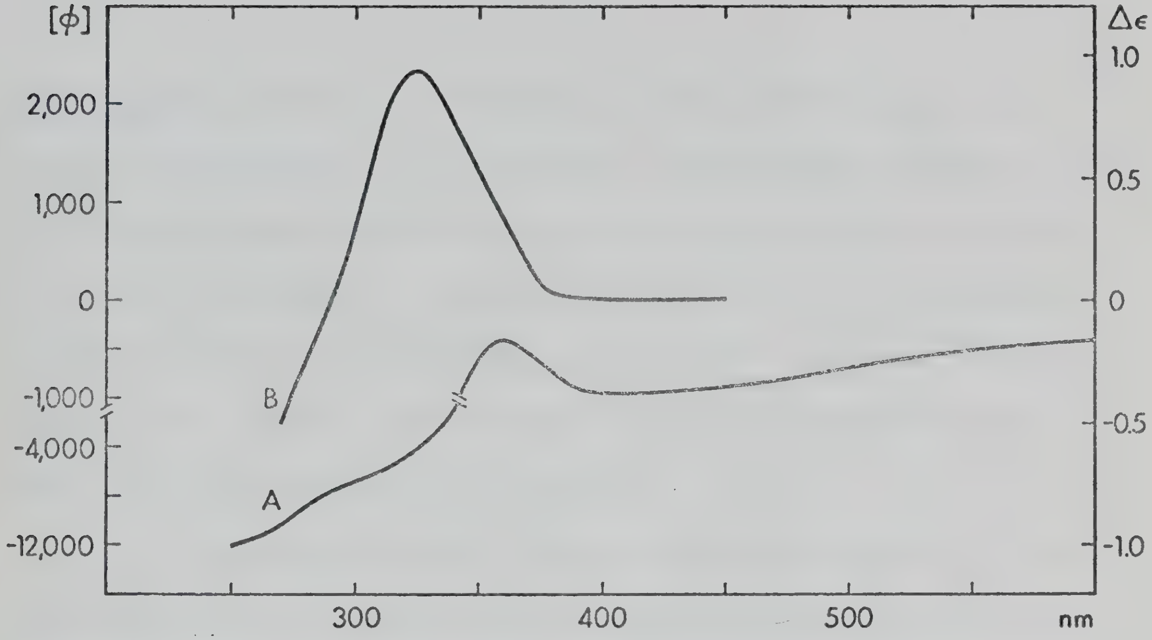


Figure 18. Chiroptical spectra of cyathin A_3 in methanol; A, ord spectrum (left scale); B, cd spectrum (right scale)

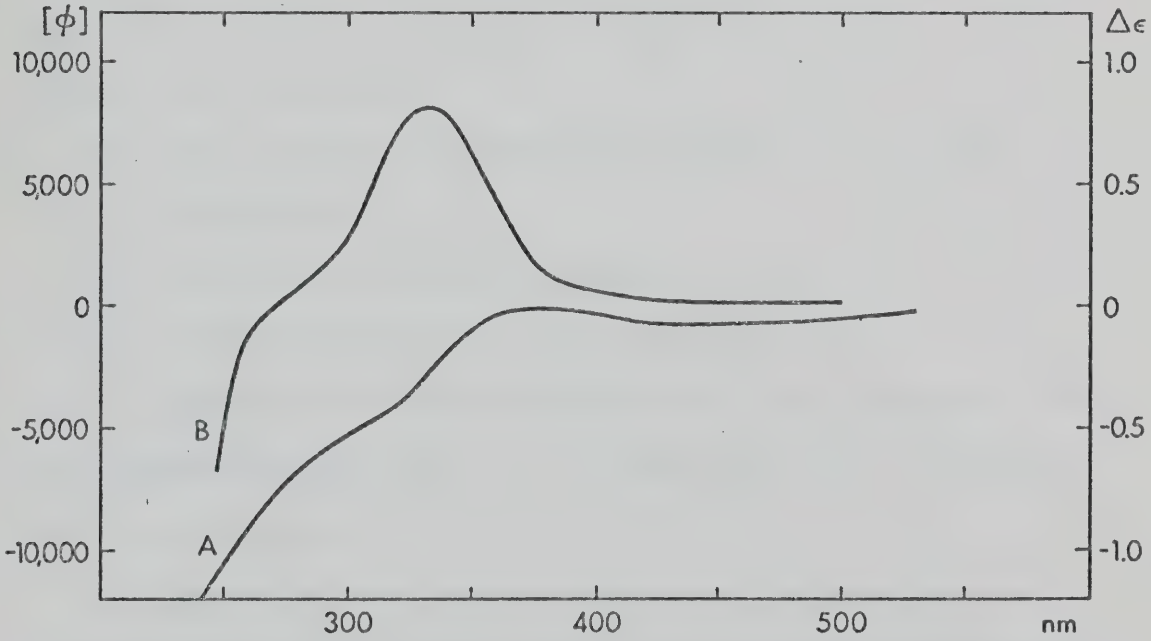


Figure 19. Chiroptical spectra of cyathin A_3 in dioxane; A, ord spectrum (left scale); B, cd spectrum (right scale)

approximately 320 nm. Positive Cotton effects are also clearly visible in the cd curves. In methanol, there is a positive maximum at 325 nm with a dichroic absorption of $\Delta\varepsilon = 1.01$ (average of three measurements); this maximum shifts to longer wavelengths in dioxane: 332 nm, $\Delta\varepsilon = 0.85$. Such Cotton effects are expected for $n-\pi^*$ transitions of α,β-unsaturated ketones[31].

No clearly defined Cotton effect ascribable to the corresponding $\pi-\pi^*$ transition can be discerned below 250 nm. It appears that in this region several chromophores give overlapping effects.

The spectral evidence discussed thus far suggests that the following functional groups are present in the cyathin A_3 molecule:

1) an α,β-unsaturated ketone group ("partially masked"),
2) one or two hydroxyl groups,
3) probably an isolated C,C-double bond.

If there are three double bonds in a molecule of the composition $C_{20}H_{30}O_3$, it follows that the molecule must be tricyclic.

Cyathin A_3 has been subjected to approximately twenty acetylation experiments (including experiments with the "A_3-mixture") under somewhat varying conditions:

1) pyridine as a solvent; approximate 25 fold excess of acetic anhydride; 16 hours at room temperature,
2) same as under 1) except reaction time shortened to two hours,
3) methylene chloride, containing a catalytic amount of pyridine, as solvent; threefold excess of acetic anhydride; one hour at room temperature, followed by ten hours at 30-40°.

There are indications from the mass spectra that complications occur under conditions 1). It was later found that conditions 2) are sufficient to bring about complete acetylation, and most of the reactions carried out in this fashion have given satisfactory results.

However, under conditions 3), the reaction may be controlled even more carefully, since it is possible to monitor the course of the reaction by analytical tlc. It appears that in the first hour at room temperature only a monoacetylated product is formed; a further ten hours at a slightly elevated temperature are necessary for complete acetylation. The R_f-values of the resulting compounds using acetone: Skellysolve B (1:3) (M) are 0.3 for the first product and 0.4 for the second. This is in line with the prediction that each consecutive acetylation decreases the polarity of the product, leading to higher R_f-values, as an acetoxyl group is usually less

strongly adsorbed than a hydroxyl group.

In order to prove that no rearrangement had occurred during acetylation, it was desirable to show that the original cyathin A_3 could be recovered upon hydrolysis of the acetylated material. Initially, the Zemplén methanolysis procedure[32] (sodium methoxide in methanol; 16 hours; room temperature) was used; again, the suspicion that cyathin A_3 might not be very stable in the presence of alkali was confirmed, in that only decomposition products were recovered. When much milder hydrolysis conditions (potassium carbonate in 80% aqueous methanol; system flushed with nitrogen; one half hour, room temperature) were used, a product was obtained whose tlc characteristics, ir and mass spectrum were identical with authentic cyathin A_3. Even milder conditions effect deacetylation of the acetyl derivative of cyathin A_3, as was later discovered accidentally: after a sample of the acetyl derivative had been left standing in methanol solution for a week, the ir spectrum lacked absorption characteristic of acetyl groups; tlc confirmed that the sample had undergone complete methanolysis.

The facile deacetylation procedure might be used to advantage for the purification of other cyathin components. A chromatographically pure cyathin component may be converted into its acetyl derivative, chromato-

graphed again and then deacetylated under the mildest possible conditions. Under favorable circumstances, contaminants still present in the original sample might either not form acetylation products at all, or form such products with different tlc characteristics, and would, therefore, be removed during the double purification process.

In fact, crystalline allocyathin B_3 has been obtained only once, after the material had been acetylated and methanolysed (accidentally), according to this procedure.

That acetylated cyathin A_3 is, in fact, a diacetyl derivative was confirmed by its mass spectrum (and other spectral characteristics) (Figure 20): the parent peak is found at m/e 402 and the corresponding ion has the composition $C_{24}H_{34}O_5$. Peaks at m/e 342 and 282 are characteristic of a O,O-diacetyl compound losing one and two molecules of acetic acid (mw 60) from the parent molecule.

O,O-Diacetylcyathin A_3 could not be induced to crystallize, but was always obtained as colorless glassy material. Further purification was attempted by sublimation (see Experimental) at 105-119°/0.02-0.2 mm; however, it is not obvious that the quality of the material was improved by this process, since no change could be detected in the ir spectrum.

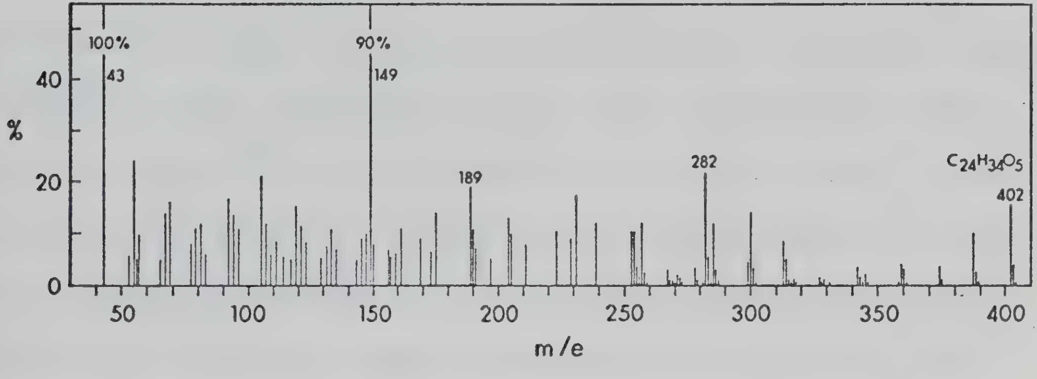


Figure 20. Mass spectrum of O,O-diacetylcyathin A_3 (Peak at m/e 149 is likely due to a phthalate impurity)

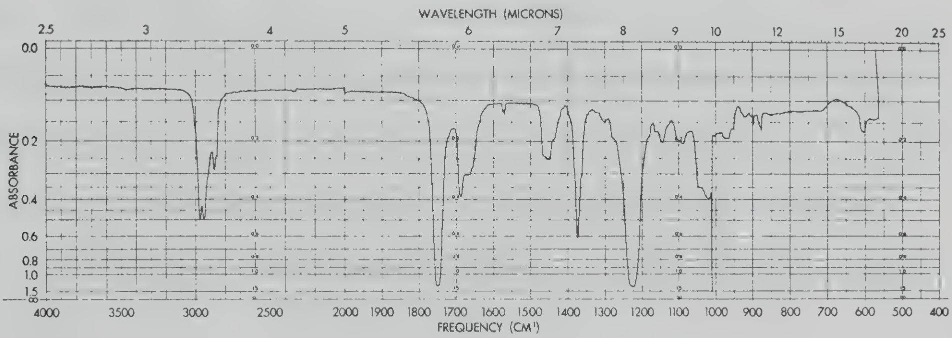


Figure 21. Ir spectrum of O,O-diacetylcyathin A_3 (CCl_4)

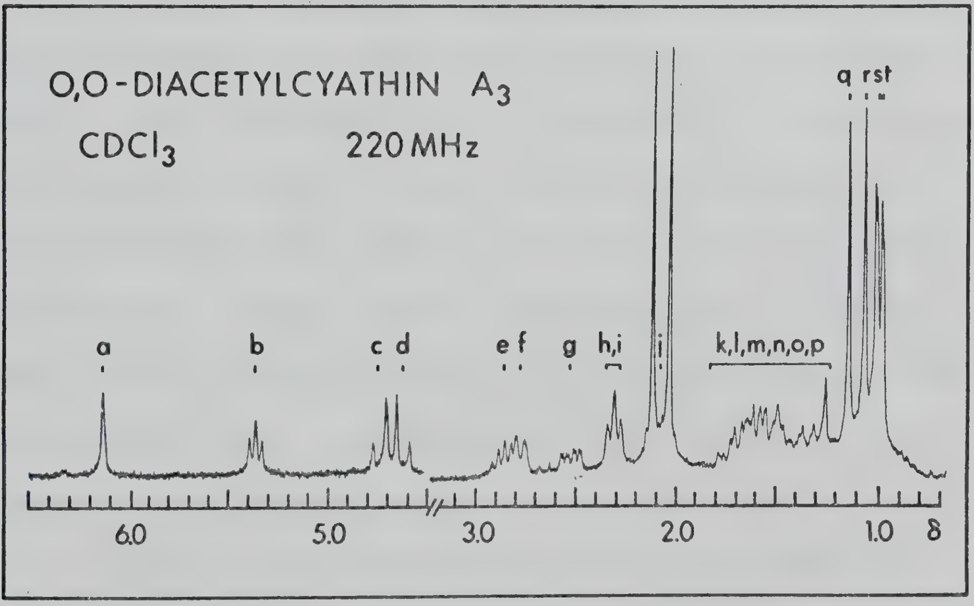


Figure 22. 220 MHz nmr spectrum of O,O-diacetylcyathin A_3 ($CDCl_3$)

The ir spectrum of O,O-diacetylcyathin A_3 (Figure 21), taken in carbon tetrachloride solution, again displays broad absorption bands; the fingerprint region (1300 - 909 cm^{-1}), in particular, is ill-defined. Tentatively, this might be attributed to conformational mobility. The spectrum reveals that the material was fully acetylated since no hydroxyl absorption remains; the very strong absorption at 1750 cm^{-1} is assigned to acetyl groups. The band which has a maximum at 1690 cm^{-1} and a shoulder at 1670 cm^{-1} is better defined than the 1650 cm^{-1} band of cyathin A_3. Undoubtably, the absorption is due to the same α,β-unsaturated ketone grouping for which there is evidence from other spectra. Still, the band is not as distinct as might be expected.

Nmr spectroscopy proved to be the most valuable source of information about the structure of O,O-diacetylcyathin A_3 and, consequently, of cyathin A_3. The spectra were measured at both 100 and 220 MHz. In most cases, deuteriochloroform was used as solvent, but benzene has also been used. Often solvent changes cause a change in chemical shift (solvent shift)[33] of certain signals and, in the present case, brought about the resolution of some of the overlapping peaks.

Nuclear magnetic double resonance (nmdr)[34] spectroscopy was used extensively to determine the inter-

actions between the various nuclei and thus to obtain information about the spatial relation of the various protons.

Some of the spectra are reproduced in Figures 22, 23, and 24. Most signals were analyzed according to first-order theory[35], although second-order analysis was applied to simple two-spin (AB) systems. The analyzed data are compiled in Table II. The signals u and v at δ 2.04 and 2.12 are readily assignable to the methyls of two O-acetyl groups (δ-values will refer to values measured in deuteriochloroform unless otherwise stated). Since cyathin A_3 does not show these signals, the derivative must be O,O-diacetylcyathin A_3, confirming the mass spectral data.

Signal a (δ 6.13) has a chemical shift characteristic of an olefinic proton; small ($\sim$1Hz), likely allylic, couplings with signals b, c and d are observed.

Signals c and d (δ 4.75 and 4.63) form an AB quartet with $J = 14$Hz, indicating a geminal relationship of the corresponding protons. The remaining couplings are small; thus no vicinal protons are indicated. The corresponding signals in cyathin A_3 appear at δ $\sim$4.2; a shift difference of 0.5 ppm is thus experienced. Signals of the carbinyl protons of a $-CH_2OH$ group typically undergo such shifts upon acetylation. The rather low δ-value

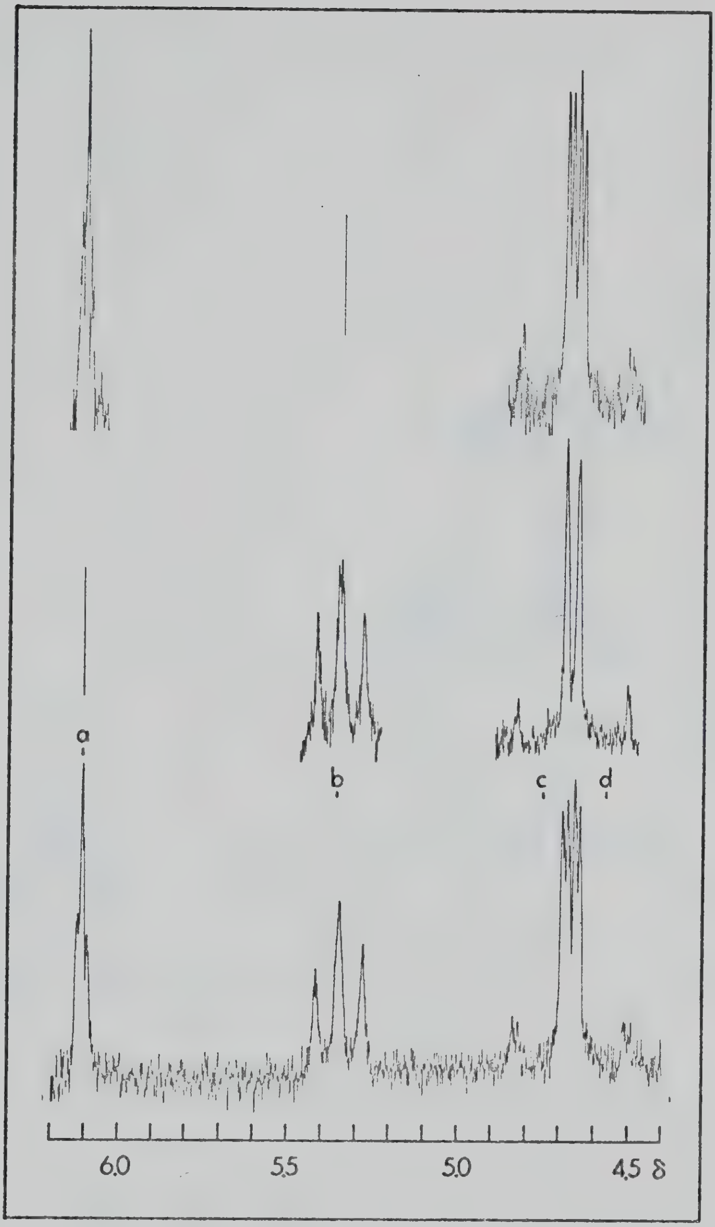


Figure 23. Low-field portion of 100 MHz nmr spectrum of O,O-diacetylcyathin A_3 ($CDCl_3$);

botton row: undecoupled

middle row: signal a irradiated

top row: signal b irradiated

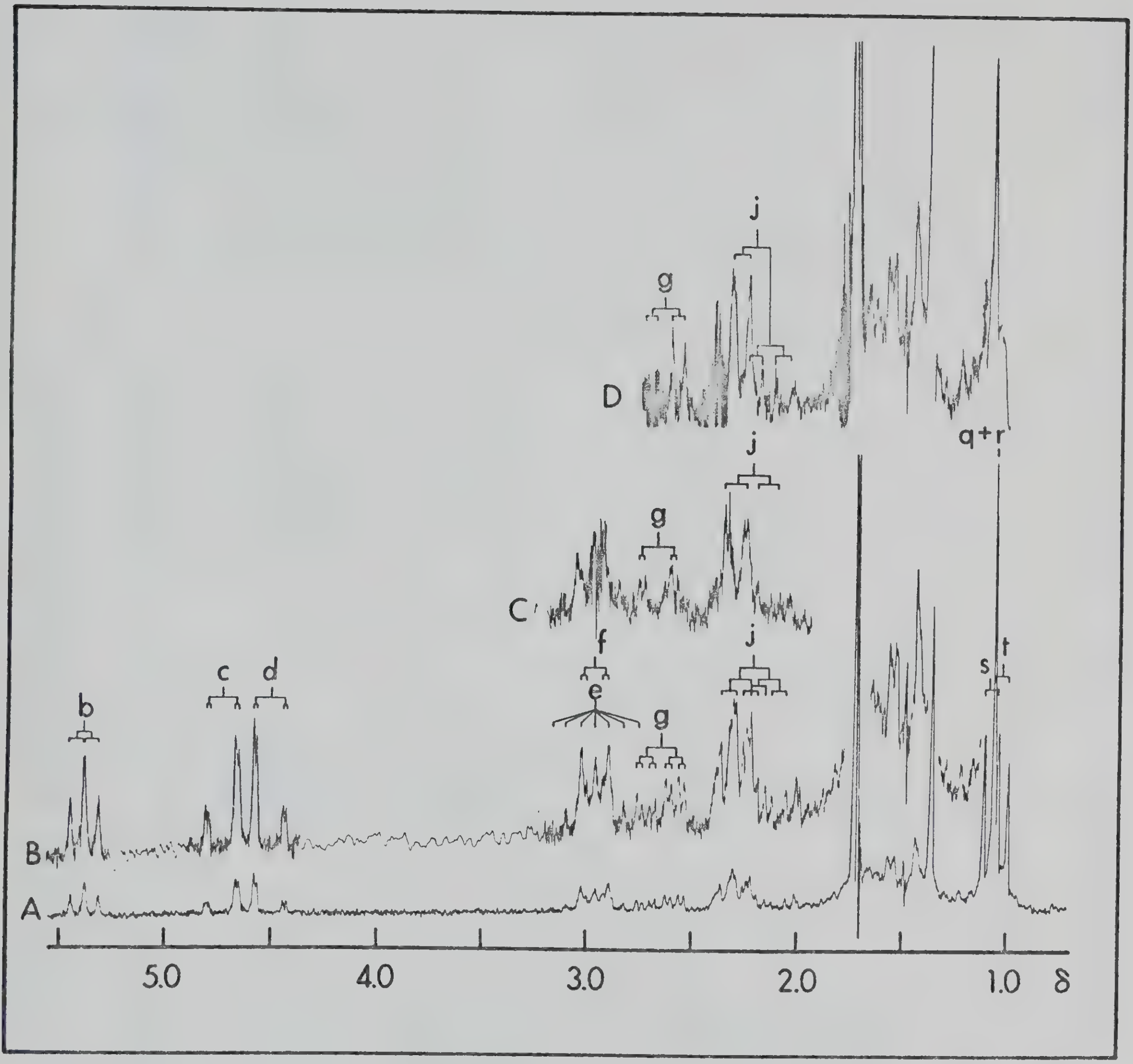


Figure 24. High-field portion of 100 MHz nmr spectrum of O,O-diacetylcyathin A_3 (C_6D_6); A, normal spectrum; B, amplified spectrum; C, signal b irradiated; D, signals e and f irradiated.

Table II. Nmr Data of O,O-Diacetylcyathin A_3

Signal	Shift in $CDCl_3$ (δ)	Shift in C_6D_6 (δ)	Multiplicity[a]	Couplings with Signals	Coupling Constants (Hz)[b]
a	6.13	-	ddd	c,d,b	1.5,1.5,0.5
b	5.36	5.37	ddddd	j,g,a,c,d	7,6,0.5,0.5,0.5
c	4.75	4.71	ddd	d,a,b	14,1.5,0.5
d	4.63	4.52	ddd	c,a,b	14,1.5,0.5
e	2.85	2.94	qq	s,t	7,7
f	2.78	2.94	dd	j,g	10,2.5
g	2.53	2.64	ddd	j,b,f	16,6,2.5
h,i	∿2.3	∿2.3	?	?	?
j	∿2.05	2.21	ddd	g,f,b	14,10,7
k,l,m, n,o,p	1.9 - 1.1	1.9 - 1.1	m	?	?
q	1.15	1.36	s	-	-
r	1.06	1.07	s	-	-
s	1.00	1.08	d	e	7
t	0.99	1.03	d	e	7
u	2.12	1.74	s	-	-
v	2.04	1.72	s	-	-

[a] For notation see Experimental.

[b] The constants are listed in the same order as the signals in the preceding column.

may be attributed to the fact that the protons are both allylic and geminal to an oxygen atom.

Signal b (δ 5.36) usually has the appearance of a broad triplet; however, in one of the spectra (Figure 23), the broad lines of the triplet are further resolved. Besides typical vicinal couplings (6 and 7Hz) with signals g and j, long-range couplings with signals a, c and d are detected. This signal ($\sim\delta$ 4.3 in cyathin A_3) has also undergone a shift of 1.1 ppm upon acetylation, thus indicating that the corresponding proton is vicinal to a secondary acetoxyl. In order to account for the low δ-value, the proton must also be allylic.

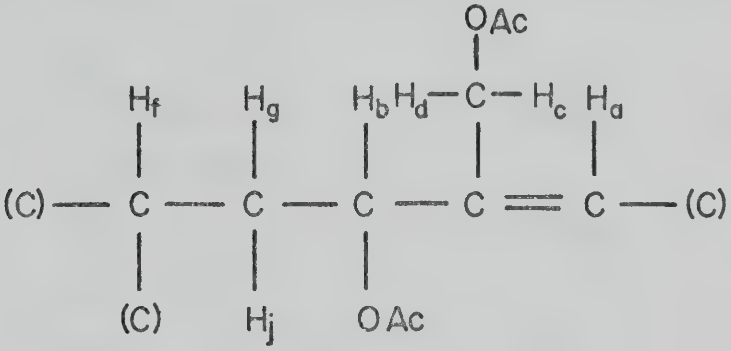
Signal g (δ 2.64) has a geminal coupling of 14 Hz (by implication, with j), a typical vicinal coupling of 6 Hz with b (already mentioned), and a third coupling of 2.5 Hz with f (which, eventually, was also identified as vicinal).

Signal f is badly overlapped with signal e, but careful inspection of the spectra shows that it is coupled to signals g and j. A coupling of 10 Hz with j suggests an axial-axial relation of the two protons and, consequently, the smaller coupling of 2.5 Hz with g is assigned to an axial-equatorial relation.

Data for signal j are obtained only by implication, since it is hidden under the acetyl signals in deuteriochloroform and overlaps with signal (h,i) in ben-

zene. Proton j has a geminal coupling of 14 Hz with g, a vicinal coupling of 7 Hz with b, and another, probably axial-axial, coupling of 10 Hz with f.

Analysis of the signals a, b, c, f, g and j thus leads to partial structure 15 in which (C) signifies a carbon atom to which no proton is attached.

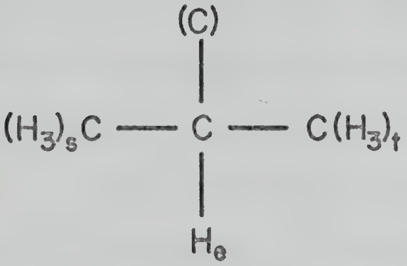


15

It should be noted that part structure 15 constitutes an isolated spin system in the sense that there is no interaction with a coupling constant $J > 0.5$ Hz with any of the remaining protons. Long-range coupling with $J < 0.5$ Hz is thus not excluded and, actually, is likely for proton f since the lines of its signal are rather broad.

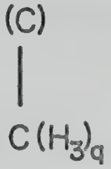
Signal e (δ 2.85) can be recognized as a septet which has typical vicinal couplings of $J = 7$ Hz (the outer lines of the signal are observed only in certain circumstances). Nmdr experiments prove that this signal

is coupled with two doublets, s and t, at δ 1.00 and 0.99. Only part structure 16, an isopropyl group, can give rise to such a coupling pattern. As no further couplings are observed for this group, it constitutes another isolated spin system. More systematically, signal e should be called a quadruplet of quadruplets (qq) as the two methyl groups which it is coupled to are not equivalent.



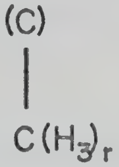
$$(H_3)_sC - \overset{(C)}{\underset{H_e}{C}} - C(H_3)_t$$

16



$$\overset{(C)}{C(H_3)_q}$$

17



$$\overset{(C)}{C(H_3)_r}$$

18

Signals q and r (δ 1.15 and 1.06) appear at positions typical of tertiary methyl groups (methyl groups attached to a quarternary carbon atom) and do not show interaction with any of the other signals. Part structures 17 and 18 emerge from these considerations.

At 220 MHz a signal corresponding to two protons, h and i, appears at approximately δ 2.3 in the form of a broad triplet. However, at 100 MHz a complex fine structure is revealed which indicates interaction with some of the remaining protons. The appearance of this signal at a rather low field suggests that the corresponding protons are allylic, but no further assignments can be made at

this stage.

The analysis has thus far accounted for 22 of the 28 skeletal hydrogens (protons of the two hydroxyl groups are not counted). The remaining six protons give rise to an unresolved group of signals (k, l, m, n, o and p) between δ 1.2 and 1.8. The location suggests that they are attached to saturated carbon atoms, and the pattern suggests that they are located on endocyclic atoms, since there are no couplings with any terminal groups (such as methyl, ethyl, methylene, etc.).

The nmr data show that the carbon skeleton of cyathin A_3 is made up of at least six exocyclic carbon atoms (isopropyl, two methyls and one hydroxymethyl). That this is the exact number cannot be proven rigorously at this stage, but it is most probable if a diterpenoid structure is anticipated. Consequently, it can be assumed that the number of endocyclic (ring) carbon atoms will be 14.

Before continuing with the discussion, it should be noted that signals e, f and (h,i) appearing below δ 2.0 are assigned to protons attached to carbon atoms α to either a C,C- or a C,O-double bond.

Uv spectra were again recorded in both a polar and nonpolar solvent (Figure 25). This time, isooctane, less polar than dioxane, was used as nonpolar solvent,

since the acetyl derivative of cyathin A_3 is soluble in this solvent, whereas cyathin A_3 itself is not.

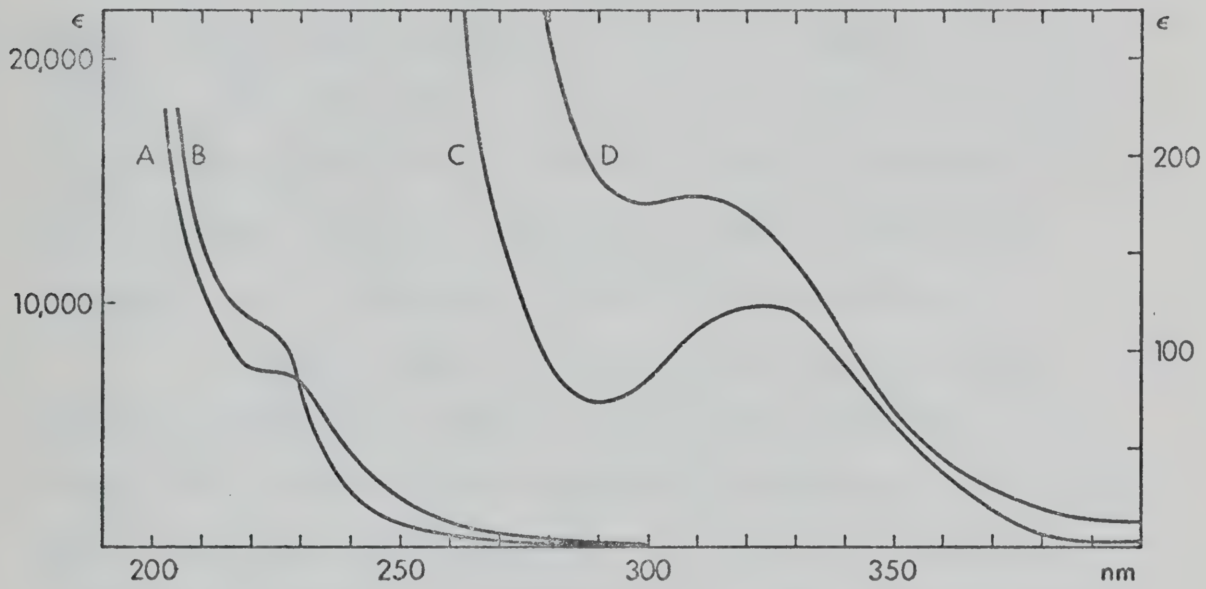


Figure 25. Uv spectra of O,O-diacetylcyathin A_3;

A, D in methanol; B, C in isooctane

A, B left scale; C, D right scale

In methanol, a shoulder is observed at λ = 230 nm with ε = 6,800. An absorption band of lower intensity has a maximum at λ = 311 nm with ε = 180. Neither absorption band, presumably due to π-π* and n-π* transitions of an α,β-unsaturated keto grouping, is well resolved. In isooctane, the resolution of the band due to the n-π* transition is improved (λ_{max} = 323 nm with ε = 130); the π-π* transition is again observed as a shoulder at λ =

225 nm with $\varepsilon = 8,700$. If the extinction values of the π-π^* transition are compared with those of cyathin A_3, it is found that they are approximately two to three times as large. The absorption due to the π-π^* transition is not suitable for comparison as it overlaps other chromophores, as yet unaccounted for. This is further indication that the carbonyl group in cyathin A_3 itself is partially masked, but is unmasked in the acetyl derivative.

The ord and cd curves of O,O-diacetylcyathin A_3 are very similar to those of the parent compound. However, the observed Cotton effects are enhanced; in particular, the positive maximum at $\lambda = 330$ nm in isooctane has a dichroic absorption of $\Delta\varepsilon = +2.73$. The comparable value for cyathin A_3 was $\Delta\varepsilon = +0.81$ (dioxane). Thus the same unmasking effect is observed.

According to Djerassi[36], the extent of ketal formation under standard conditions (addition of a small amount of hydrochloric acid to a methanolic solution of the sample) indicates the size of the ring in which a keto group is located. The following rules were established: a cycloheptanone will form the ketal to the extent of 21%; a cyclohexanone, to 93%; and a cyclopentanone, to 24%. In Djerassi's work, ord measurements of the Cotton effect due to the n-π^* transition permitted determination of the extent of ketalization.

Uv or cd measurements (or, in general, any spectroscopic method which permits measurement of carbonyl absorption in methanol solution, and in which addition of the acidic reagent does not interfere with the measurement) should likewise allow the detection of ketal formation. The n-π^* transition of the keto group in cyathin A_3 gives rise to a well-defined cd maximum at 325 nm in methanol. In the uv and ord curves, this feature is less well defined. The cd method was therefore chosen to follow the extent of ketalization. However, the experiment was undertaken with reservation since Djerassi also reported that α,β-unsaturated ketones in the few cases investigated did not form ketals. The results obtained thus came as a pleasant surprise. When a small drop of methanol saturated with hydrogen chloride was added to 1 ml of a 0.2% solution of cyathin A_3 in methanol, the positive Cotton effect at 325 nm disappeared very rapidly. As the reaction course was continuously monitored by cd measurements, the following conversion times could be computed:

$$t_{50\%} = 2 \text{ min}$$

$$t_{90\%} = 8 \text{ min}$$

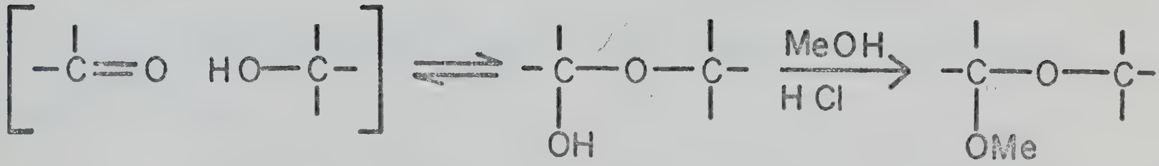
$$t_{99\%} = 18 \text{ min}$$

After complete ketalization the observed ord curve is plain negative (at least down to 250 nm); the uv spectrum, likewise, shows no maximum above 300 nm.

When the small amount (∿2 mg) of material used was isolated and its ir spectrum measured in carbon tetrachloride solution, the absorption at 1650 cm^{-1} had also disappeared, the fingerprint region was surprisingly sharp, and the intensity of the hydroxyl absorption appeared diminished when compared with that of the starting material. Obviously, cyathin A_3 had been dramatically affected.

The molecular weight and formula were also determined on this small sample by hrms. The mw was 332 (an increase of 14 mass units when compared with the mw of cyathin A_3), and the composition was $C_{21}H_{32}O_3$ $(+CH_2)$. Thus, the net reaction was one of methylation, and corresponded to the transformation of a hemiketal to a half methyl ketal.

It was now abundantly clear that the "masking" effect mentioned in so many previous instances could be explained in terms of hemiketal formation. In solution, cyathin A_3 exists in two forms: a hydroxyketone **19** and a hemiketal **20**, the two forms being in dynamic equilibrium. In methanolic hydrochloric acid, a ketal **21** is formed irreversibly.


19 20 21

The ketalization experiment was repeated on an enlarged scale (20 mg of cyathin A_3). After work-up, the product appeared to be homogeneous (according to tlc) and was used without further purification.

Under aqueous conditions, ketals are susceptible to hydrolysis. In order to confirm the ketal nature of the material, to test its sensitivity to aqueous acid, and in order to recover cyathin A_3 (and thus show that no rearrangement had occurred), hydrolysis experiments were carried out in the system acetone-water-hydrochloric acid (20:2:1). Preliminary tests showed that complete hydrolysis could be effected under either of the following conditions: two hours at ∿50°, or 16 hours at 32°. When a 40-mg sample was subjected to the latter conditions, a mixture of products was obtained. After chromatographic purification, 11 mg of cyathin A_3 was recovered and identified by its crystallinity, tlc characteristics and ir spectrum.

Considering the more vigorous reaction conditions and the low yield (∿25%), the hydrolysis is clearly

not as smooth as the ketal formation. This suggests that the ketal form of cyathin A_3 is more stable to acid than the keto form.

No special effort was made to crystallize the material and the ketal was handled as a gum.

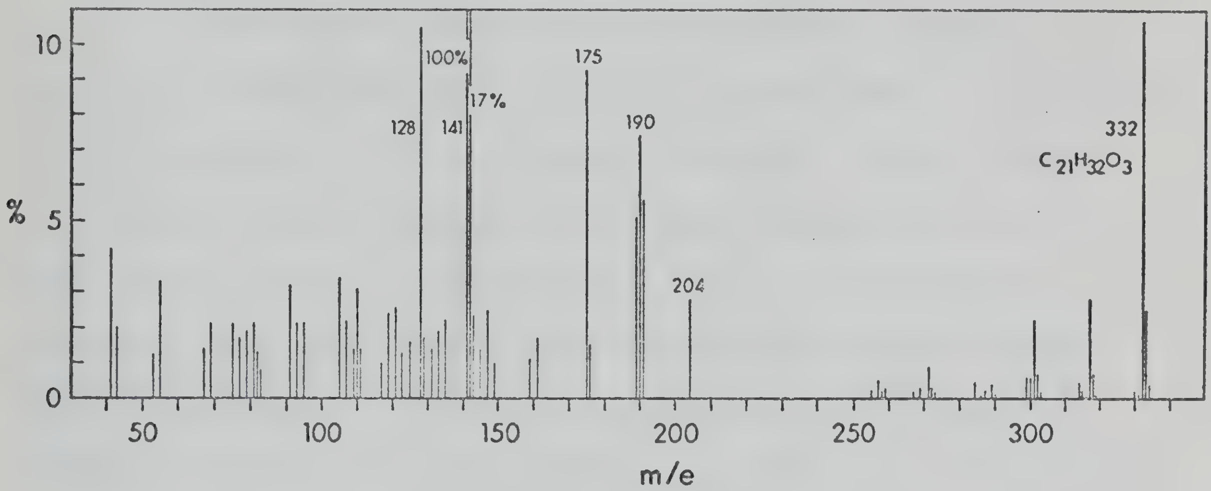


Figure 26. Mass spectrum of cyathin A_3 methyl ketal

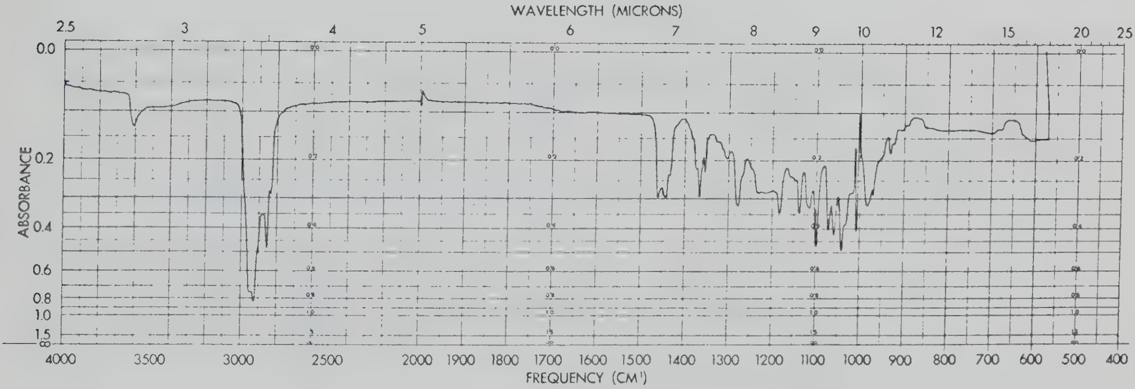
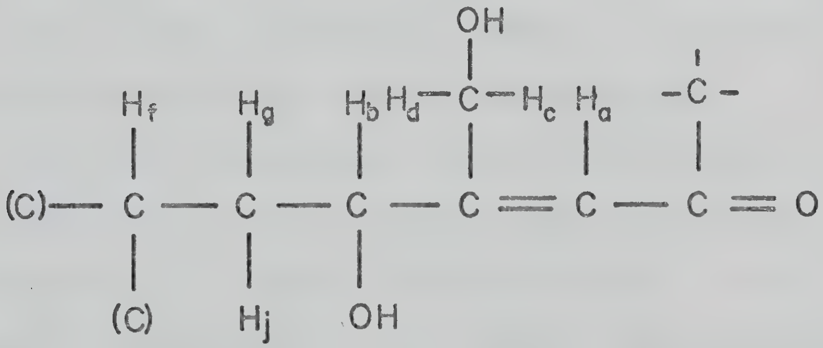


Figure 27. Ir spectrum of cyathin A_3 methyl ketal (CCl_4)

The mass spectrum (Figure 26), briefly mentioned above, had a very intense base peak at m/e 141 as its most remarkable feature. All other mass peaks have

an intensity of less than 15% of the base peak. The corresponding ion had the composition $C_7H_9O_3$. For the first time in any of the mass spectra in the cyathin series, a fragment of mass less than 200 containing oxygen was observed.

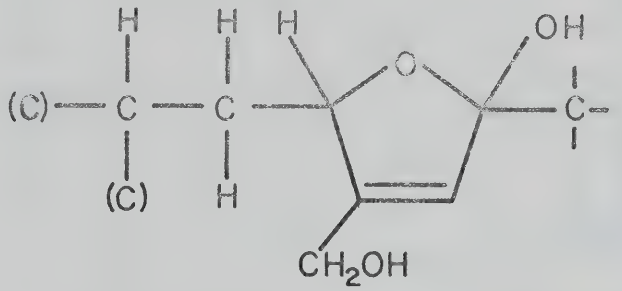
Assuming that no rearrangement had taken place, it can be concluded that the three oxygen atoms of cyathin A_3 are located on three of seven adjacent carbon atoms. Two oxygens are accounted for in part structure 15 and the third oxygen is incorporated in an α,β-unsaturated ketone. The only logical way to place the third oxygen is in the form of a keto group attached to the carbon carrying proton a. Part structure 15 can thus be expanded to 22:



22

If 22 is incorporated in a six- or seven-membered ring, the double bond must have a cis configuration. For geometric reasons, the ketone cannot form a

hemiketal with the primary but only with the secondary hydroxyl group. Hence, part structure 23 represents the hemiketal form of cyathin A_3:



23

As mentioned above, the only functional group indicated (in the ketal) by the ir spectrum (Figure 27) is a hydroxyl group (3610 cm^{-1}). It should be noted, however, that the methyl ketal, in contrast with the diacetyl derivative or cyathin A_3 itself, exhibits well-defined bands in the fingerprint region; a sign that the conformation of this derivative is more rigid.

100 MHz nmr spectra of the ketal (Figures 28 and 29) were obtained in deuteriochloroform and benzene solution. Comparison of Figure 28 with Figure 29 very clearly shows how the resolution of the signals improves when the spectrum is taken in benzene. Again a number of double irradiation experiments were carried out to determine the relationship of the various signals.

The nmr data are listed in Table III. The sig-

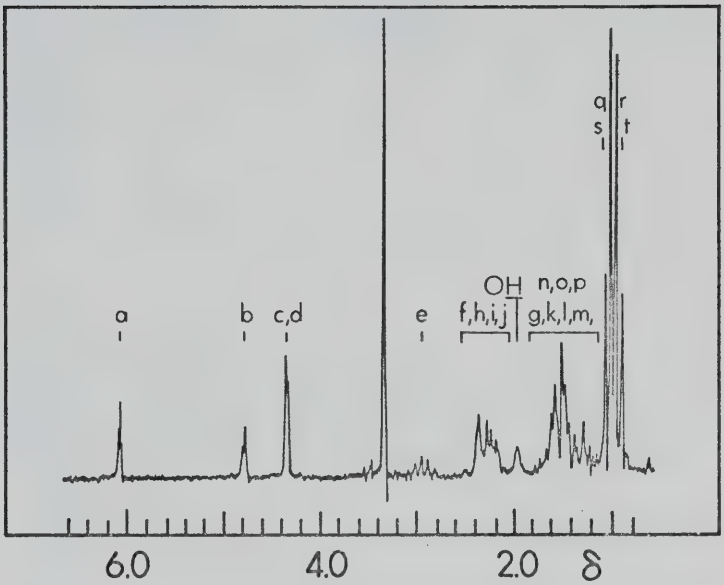


Figure 28. 100 MHz nmr spectrum of cyathin A_3 methyl ketal ($CDCl_3$)

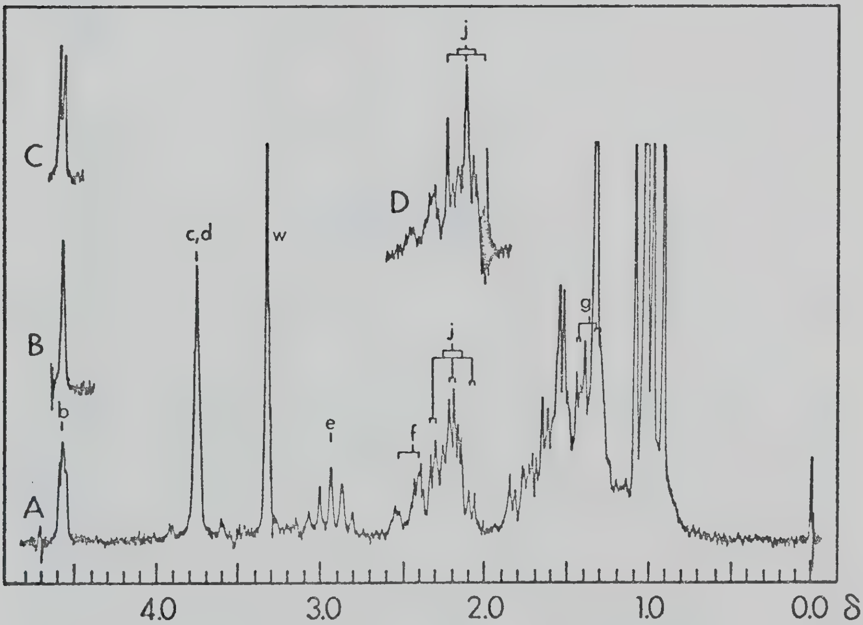


Figure 29. 100 MHz nmr spectrum of cyathin A_3 methyl ketal (C_6D_6);

A, undecoupled; B, signal j irradiated;

C, signal g irradiated; D, signal b irradiated

Table III. Nmr Data of Cyathin A_3 Methyl Ketal and O-Acetylcyathin A_3 Methyl Ketal

Signal	Shift in $CDCl_3$ (δ)	Shift in C_6D_6 (δ)	Multiplicity	Couplings with Signals	Coupling Constants (Hz)	Shift of Acetyl Derivative in C_6D_6 (δ)
a	6.06	5.86	dd	c,d	1.5,1.5	5.88
b	4.79	4.60	u	(j,?)	(3,?)	4.56
c	4.34	3.76	u	(d,a)	(16,1.5)	4.49
d	4.34	3.76	u	(c,a)	(16,1.5)	4.32
e	2.95	2.96	qq	s,t	6.5,6.5	2.94
f	∿2.42	2.44	(dm)	(j,g,?)	(12,?)	2.43
g	∿1.4	1.35	(dd)	(j,f)	(12,?)	∿1.4
h,i	∿2.2	2.20	?	?	?	2.2
j	2.25	2.19	ddd	g,f,b	12,12,3	2.20
k,l,m, n,o,p	1.8 - 1.2	1.9 - 1.2	?	?	?	1.9 - 1.2
q	1.01	1.31	s	-	-	1.28
r	0.97	1.01	s	-	-	1.02
s	1.03	1.04	d	e	6.5	1.03
t	0.93	0.93	d	e	6.5	0.91
u ($O_2CC\underline{H}_3$)	-	-	-	-	-	1.63
w ($OC\underline{H}_3$)	3.31	3.34	s	-	-	3.28

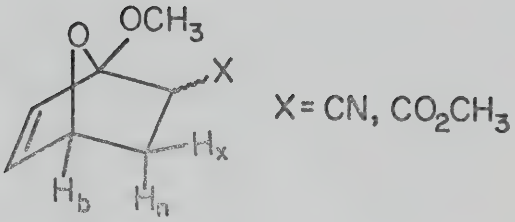
u = unresolved, ( ) = implied, ? = unknown

nals are labelled, where possible, with the same letter used in the nmr analysis of O,O-diacetylcyathin A_3. Chemical shift, coupling pattern and, to a lesser extent, intensity of the signals served as characteristics for identification. No attempt was made to distinguish between the methyl signals q and r or between s and t; these signals are merely listed in the order of descending δ-values. More serious difficulties were encountered in the assignments of signals f, g, and j. One of the three is buried in the signal heap between δ 1.2-1.8, while the two others overlap each other and signal (h,i). Tentatively, the following assignments are made: the signal at δ 2.25 showing a coupling pattern of J = 12, 12, 3 Hz is ascribed to proton j, since this pattern is reminiscent of signal j in O,O-diacetylcyathin A_3 (J = 14, 10, 7 Hz); the broad doublet at δ 2.42, a shift typical of an allylic proton, is labelled f, since proton f is further removed from the centre of change and its signal would not be expected to alter drastically; by default, the hidden signal at δ 1.4 is assigned to proton g. (The δ-values refer to the spectra measured in deuteriochloroform solution).

The olefinic protons, the methylene protons of the primary alcohol, and the isopropyl and tertiary methyl protons undergo little change and their signals are recognized without difficulty.

Signals b, g, and j are most affected by ketal formation. Signal b is a relatively narrow band (width at half-height 6 Hz, as compared with 14 Hz in the case of O,O-diacetylcyathin A_3) showing unanalyzable fine structure. Nmdr experiments, however, show that the coupling of b with j is 3 Hz and with g less than 1 Hz. If the above analysis is correct, signal j undergoes a small downfield shift of ∿0.2 ppm and signal g a more substantial upfield shift of ∿1.1 ppm. (The shift values were obtained from the spectra determined in deuteriochloroform solution.) Signal f also experiences an upfield shift of ∿0.4 ppm but its coupling cannot be fully analyzed.

The nmr data confirm that it is the secondary hydroxyl group that participates in the ketalization. The changes in coupling pattern experienced by signals b, g, and j are in full agreement with the proposal that ketalization creates a bridged system in which proton b is attached to a bridgehead carbon. The coupling constants are quite similar to those observed in model compound **24**, in which $J_{b,x} = 4.5$ and $J_{b,n} = 0$ Hz.[37] Similar couplings between bridgehead proton and endo and exo protons are observed in the norbornene series (ref 35a, p 289).



24

According to this model, proton j is in a quasi-exo and proton g in a quasi-endo configuration.

The acetyl derivative of cyathin A_3 methyl ketal was prepared in order to obtain further evidence that the secondary hydroxyl group was involved in ketal formation. Again, very mild conditions were used for acetylation: a dilute methylene chloride solution of the ketal, containing a small amount of pyridine, was treated with an approximately sixfold excess of acetic anhydride, first at 26° for 1.25 hours and then at 40° for one hour. By monitoring the course of the reaction by tlc, it was observed that most of the starting material was consumed after one hour at 26°. The extremely mild conditions are the first indication that the hydroxyl group undergoing acetylation is primary. After work-up the product was used directly for spectral determination since tlc indicated a high degree of purity.

The mass spectrum (Figure 30) indicated that a monoacetyl derivative had formed. The molecular weight

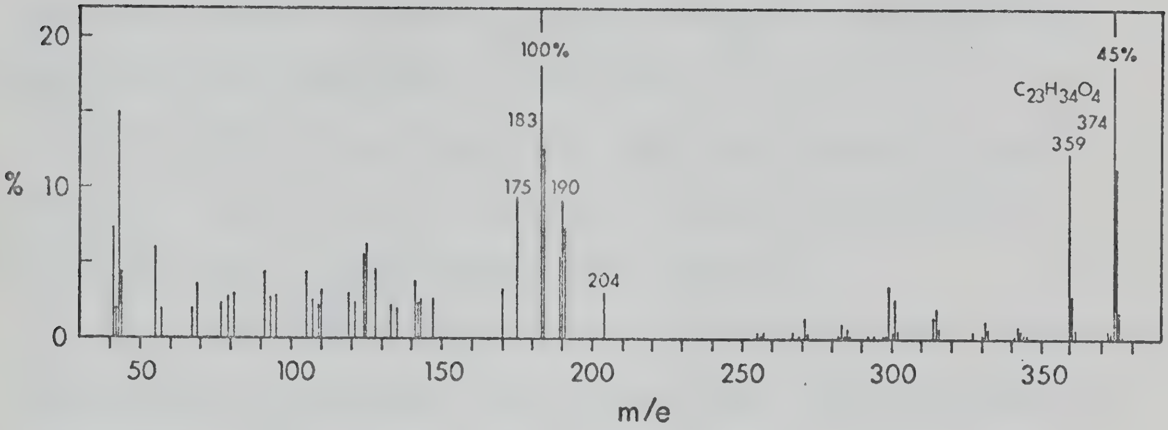


Figure 30. Mass spectrum of O-acetylcyathin A_3 methyl ketal

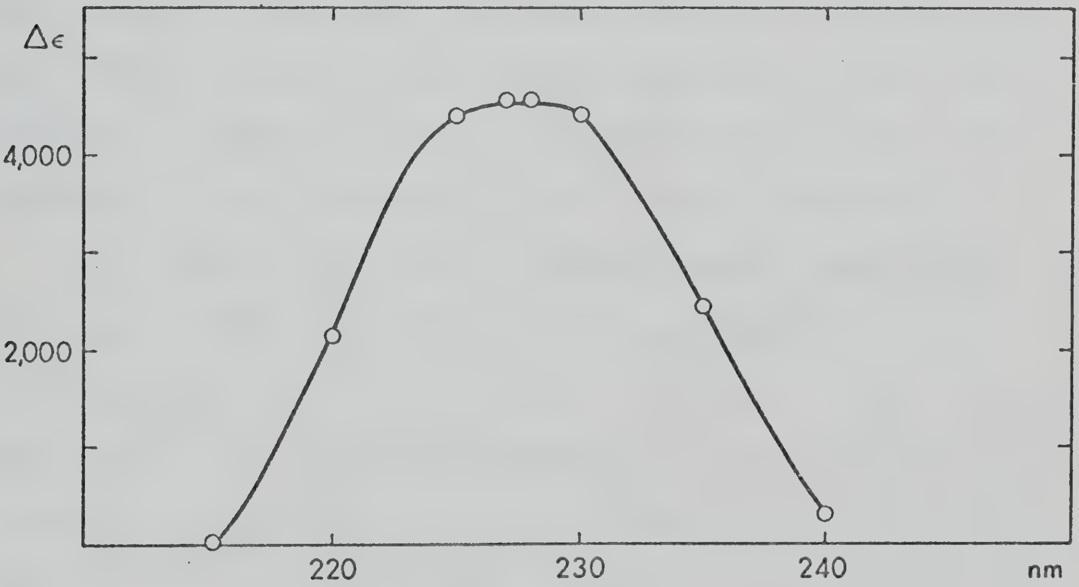


Figure 31. Computed uv difference spectrum between O,O-diacetylcyathin A_3 and O-acetylcyathin A_3 methyl ketal (isooctane)

had increased to 374 (+42, when compared with the ketal), and the composition was $C_{22}H_{34}O_4$ ($+C_2H_2O$). As expected, the base peak had also shifted from m/e 141 to 183.

The ir spectrum now lacked absorption in the hydroxyl region, but an absorption band characteristic of an acetoxyl group appeared at 1745 cm^{-1}.

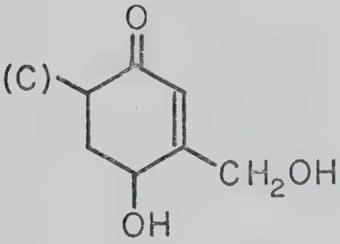
The nmr spectrum (again measured in both deuteriochloroform and benzene, but values cited refer to deuteriochloroform) remained unchanged (Table III), except that the signal for the hydroxyl proton at δ 1.98 had disappeared, a typical three-proton signal for an acetyl group appeared at δ 2.11, and the signal for the methylene protons of the primary alcohol group underwent a downfield shift of 0.4 ppm, a typical "acylation shift" for primary alcohols. Thus, structure 23 is further confirmed.

The uv, ord and cd curves remain essentially unchanged. The uv spectrum of O-acetylcyathin A_3 methyl ketal, however, was more closely investigated and compared with that of O,O-diacetylcyathin A_3 in order to obtain a difference spectrum, i.e. a spectrum in which the differences of the extinction coefficients of the two compounds is plotted versus wavelength (Figure 31).

This difference spectrum suggests that the absorption band of the π-π* transition of the enone chromophore of O,O-diacetylcyathin A_3 has its maximum at 228

nm with $\varepsilon = 4,400$. According to the Fieser rules (ref 35b, p 160) a β,β-disubstituted cyclohexenone should have an absorption maximum at 239 nm (base value of six-membered cyclic enone (215) + 2 β-substituents (2 x 12)), which is reduced to 228 nm after appropriate solvent correction. Thus part structure 22 could be contained in the six-membered ring of part structure 25. However, the low extinction value mitigates against this (ε_{max} usually 10,000-20,000)[38].

The mass spectral data also would not support part structures 25 for the ketone or 26 for the ketal, since genesis of 27, suggested for the m/e 141 fragment (see below), would involve a highly improbable carbene-forming cleavage.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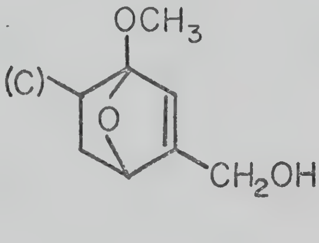
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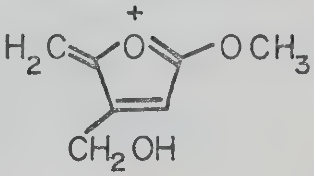
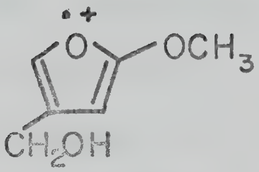

27

On the other hand, one would expect that 26 would undergo a retro Diels-Alder fragmentation and give rise to the presumably stable fragment 28 (m/e 128, $C_6H_8O_3$) which should constitute the base peak.



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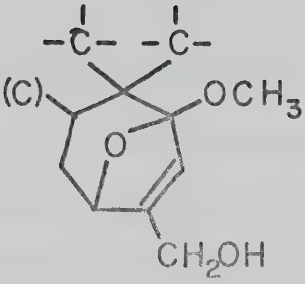
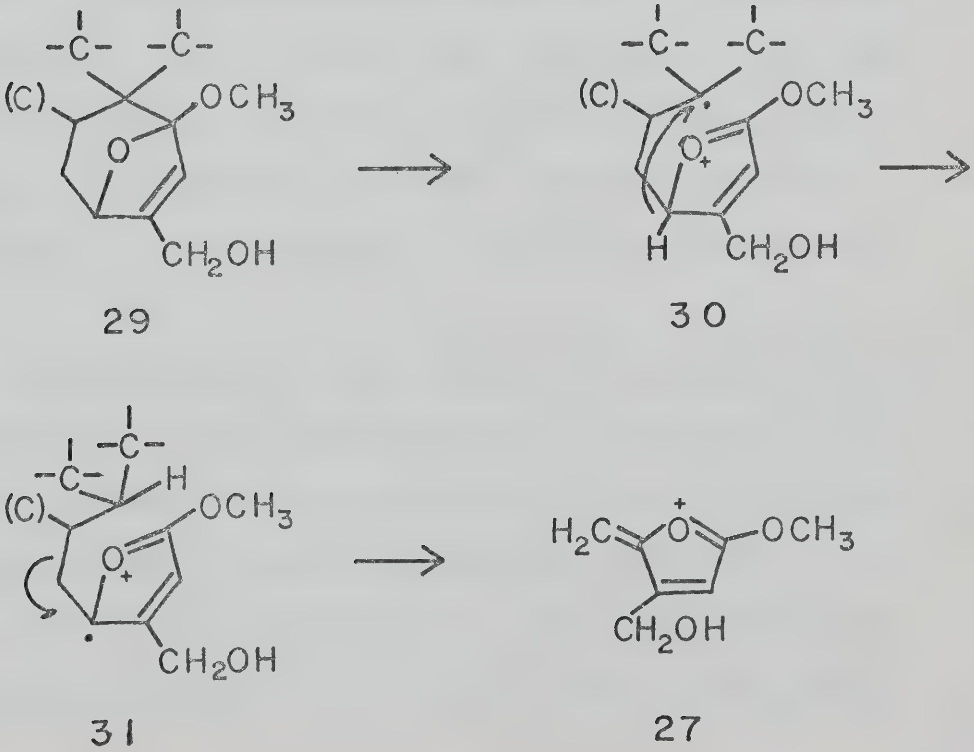


29

Since this peak has an intensity of only 10%, a seven-membered structure such as 29 is more probable, although rings of even larger size cannot be excluded at this stage. Fragmentation of a molecule in which 29 is incorporated as a part structure can be rationalized according to Scheme III.

Scheme III



3) Allocyathin B_3

The investigation of allocyathin B_3 was modelled after that of cyathin A_3. Skills learned in the handling of cyathin A_3 resulted in fewer experimental mishaps; experiments were carried out on allocyathin B_3 only after analogous experiments with cyathin A_3 had been thoroughly studied; interpretation of the spectra of allocyathin B_3 was facilitated by virtue of the fact that the corresponding spectra of cyathin A_3 had been previously analyzed, and it was assumed that the compounds were closely related.

The experimental work with allocyathin B_3 proved more laborious, however, since less material was available and it appears to be less stable. The increased sensitivity is presumably attributable to the additional functional site, a C,C-double bond. Conversely, the additional functionality yields new information useful for the structure determination of both cyathin compounds, since cyathin A_3 is anticipated to be dihydroallocyathin B_3.

As mentioned before, allocyathin B_3 was obtained in crystal form on only one occasion. In numerous recrystallization attempts on this one crop, crystals could not be induced to grow to sizes of the order of 1 mm^3; they were always microscopically small. The melting point of a recrystallized sample was 143-144°. Most of the work,

however, was carried out with noncrystalline material.

The molecular formula, $C_{20}H_{28}O_3$ (mw 316), was determined by mass spectrometry (Figure 32). The fragmentation pattern is similar to that of cyathin A_3, except that all m/e values are reduced by 2. This indicates that the additional double bond is located in that part of the molecule remote from the oxygen atoms.

The ir spectrum (Figure 33) taken in chloroform solution differs from that of cyathin A_3 only in the following respects: the "carbonyl absorption" at 1650 cm^{-1} appears as a slightly sharper band; very weak bands between 3000 and 3100 cm^{-1} (much better defined in the allocyathin B_3 derivatives) are attributed to the stretching vibration of protons situated on a C,C-double bond.

The solid-phase spectrum (Figure 34) differs more drastically; the predominant feature is the strong, sharp carbonyl absorption band at 1650 cm^{-1}, indicating that allocyathin B_3 crystallizes in the keto form. The differences in crystallization behavior mentioned above are perhaps attributable to this fact. It might also be concluded that the ketal is the more stable form of cyathin A_3 but the less stable one of allocyathin B_3, or this may be simply a question of solubility.

The nmr spectrum again was poorly defined and will not be discussed here, since the spectra of the deri-

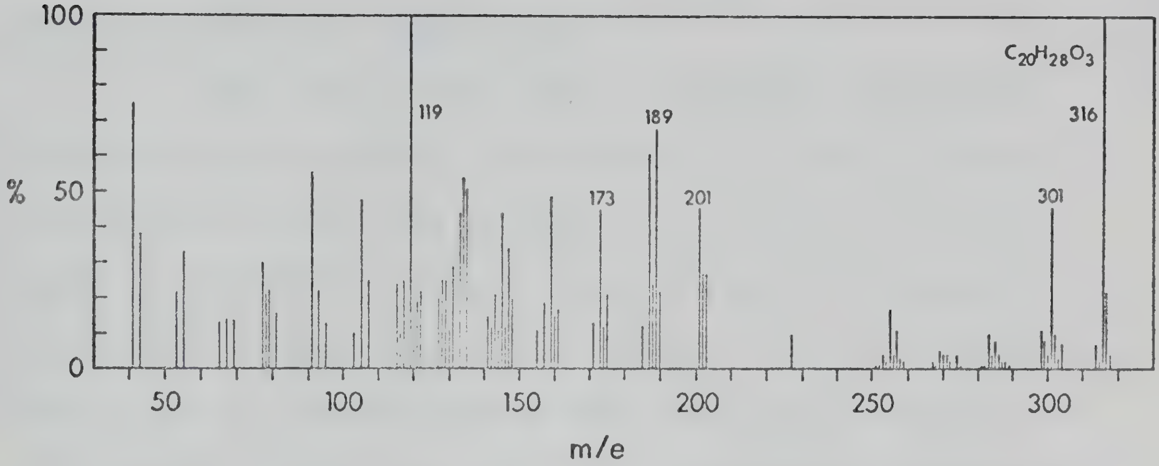


Figure 32. Mass spectrum of allocyathin B_3

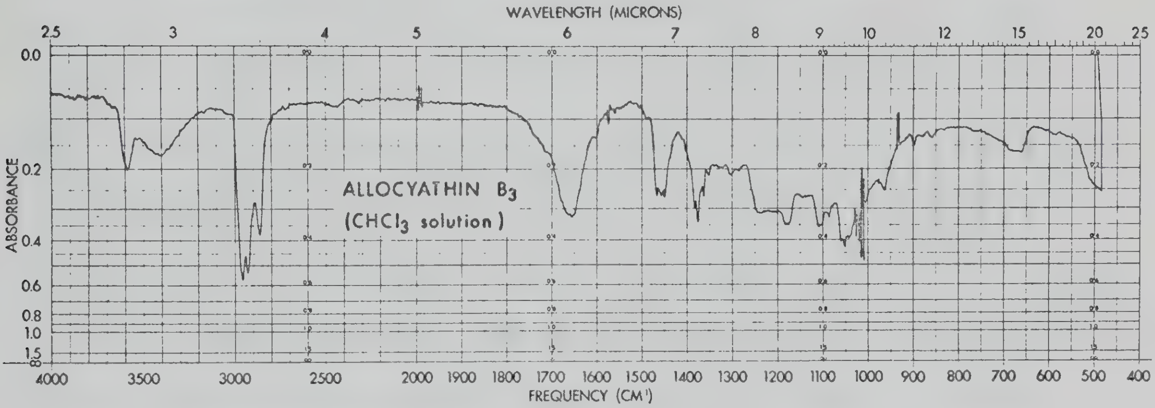


Figure 33. Ir spectrum of allocyathin B_3 ($CHCl_3$)

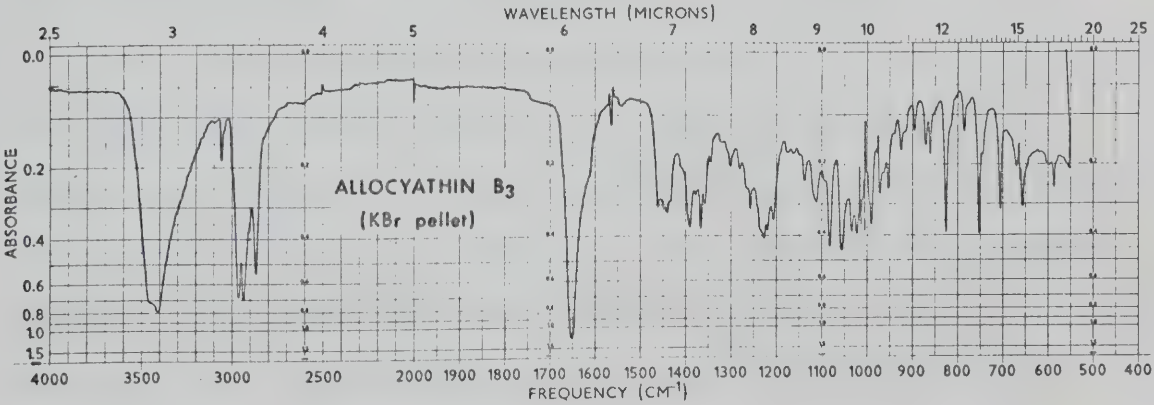


Figure 34. Ir spectrum of allocyathin B_3 (KBr pellet)

vatives yield more information.

The uv spectrum, taken in methanol, displays a well-defined maximum at 235 nm with ε = 5,000; no shoulders or weak maxima are detected. At first sight, this seems to indicate the presence of a single chromophore, possibly a conjugated diene or enone. However, close inspection of the spectrum reveals that this band is of non-Gaussian shape, that there is considerable absorption in the region 260-280 nm ($\varepsilon_{275\ nm}$ = 1,600 compared with $\varepsilon_{276\ nm}$ = 130 in the case of cyathin A_3), and, since the observed extinction coefficient is 100 at 325 nm, that a band of low intensity might be hidden above 300 nm. These facts suggest the presence of more than one chromophore: by chance, the overlapping bands produce only one maximum.

Above 250 nm, the ord curve displays a positive Cotton effect superimposed upon a plain negative curve. In the cd curve, the same Cotton effect is undisturbed by other chromophores and is observed as a positive maximum at 327 nm with a dichroic absorption of $\Delta\varepsilon$ = +1.6. These are clear indications of the n-π^* transition of an α,β-unsaturated ketone; the corresponding absorption remained undetectable in the uv spectrum. The increase in dichroic absorption (as compared with cyathin A_3, where $\Delta\varepsilon$ = +1.0) again points to a predominance of the keto form in the tautomeric equilibrium.

The acetyl derivative was prepared in several separate experiments using each time a large excess of acetic anhydride-pyridine reagent at room temperature for two hours. A total of 24 mg of pure product (according to tlc on argentated silica gel) was obtained from 50 mg of chromatographically pure allocyathin B_3. The rather poor yield (38%) from such a simple reaction indicates that either the starting material was still impure, or that the reaction conditions lead to a considerable number of side reactions.

Mass and ir spectra of this derivative are not available at present, since the sample which was designated for these measurements was allowed to stand for too long a time in methanol. This resulted in deacetylation as mentioned above.

Chiroptical and uv measurements were carried out in methanol. As the rate of methanolysis is unknown, it is possible that at least partial deacetylation occurred. Consequently, these spectra must be interpreted with caution. Measurement in non-hydroxylic solvents would circumvent this problem, but because of the limited amount of material available this has not been done.

The same nmr measurements and evaluations as described for O,O-diacetylcyathin A_3 were undertaken for O,O-diacetylallocyathin B_3 (Figures 35, 36; Table IV).

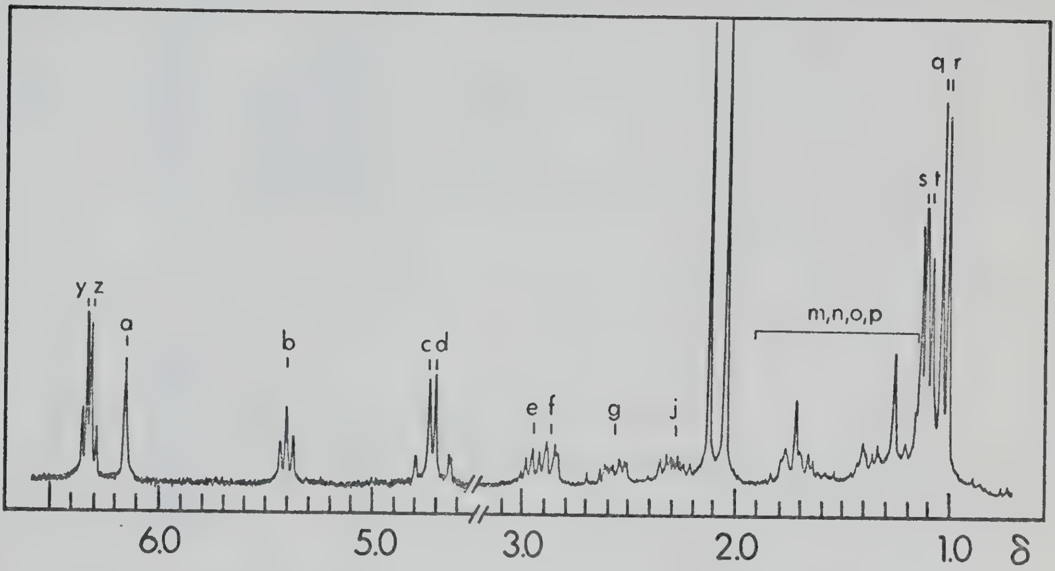


Figure 35. 220 MHz nmr spectrum of O,O-diacetylallocyathin B_3 ($CDCl_3$)

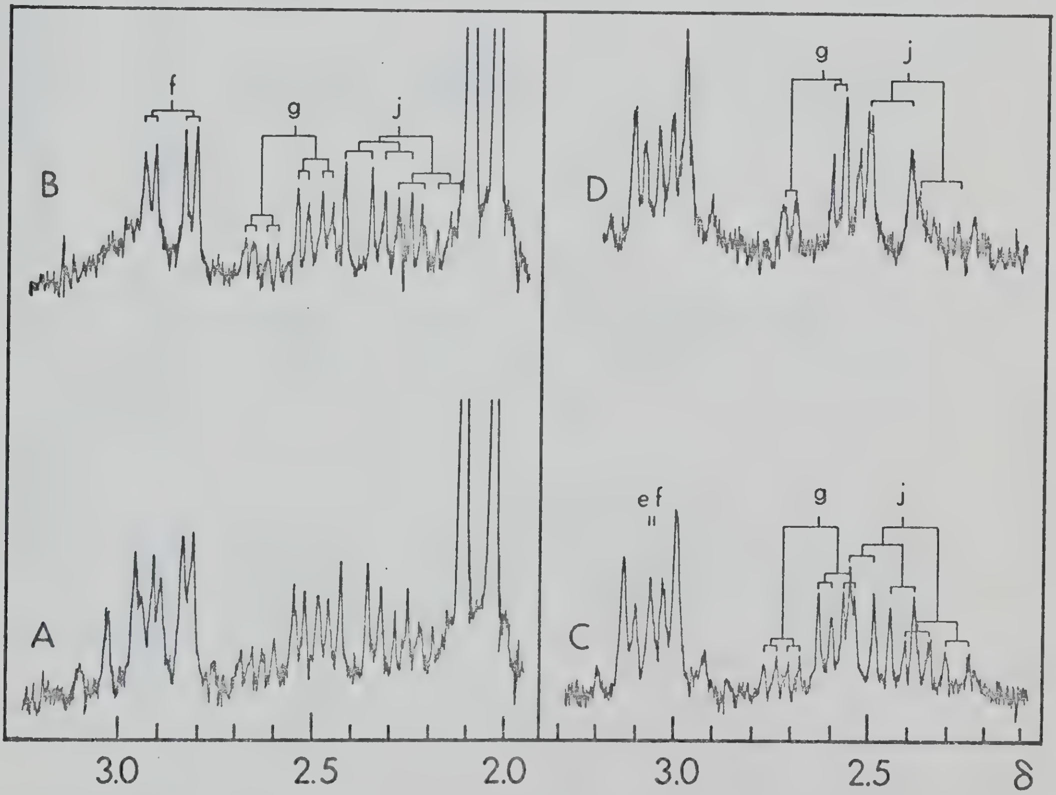


Figure 36. Portions of 100 MHz nmr spectra of O,O-diacetylallocyathin B_3

A, undecoupled ($CDCl_3$); B, signals s and t irradiated (see Fig. 35); C, undecoupled (C_6D_6); D, signal b irradiated.

Table IV. Nmr Data of O,O-Diacetylallocyathin B_3

Signal	Shift in $CDCl_3$ (δ)	Shift in C_6D_6 (δ)	Multiplicity	Couplings with Signals	Coupling Constants (Hz)
a	6.15	-	d,d	c,d	1.5,1.5
b	5.39	5.41	d,d	g,j	6.5,6.5
c	4.75	4.74	d,d	d,a	14.5,1.5
d	4.68	4.64	d,d	c,a	14.5,1.5
e	2.96	3.06	q,q	s,t	7,7
f	2.87	3.06	d,d	j,g	10.5,3
g	2.56	2.63	d,d,d	j,b,f	14,6.5,3
j	2.27	2.41	d,d,d	g,f,b	14,10.5,6.5
m,n,o,p	1.9 - 1.1	1.9 - 1.2	?	?	?
q	1.04	1.22	s	-	-
r	1.00	1.02	s	-	-
s	1.12	1.20	d	e	7
t	1.10	1.14	d	e	7
u	2.12	1.74	s	-	-
v	2.05	1.72	s	-	-
y	6.34	-	d	z	5.5
z	6.31	-	d	y	5.5

The same labelling system has again been used so that structurally related protons of both derivatives are assigned identical letters.

Signals a, b, c, d, e, q, r, s, and t are readily recognized by their chemical shift and coupling pattern, and are assigned in the same manner as outlined for O,O-diacetylcyathin A_3.

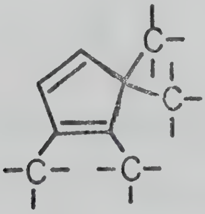
Signals y and z which form an isolated AB quartet at δ 6.34 and 6.31 (δ-values again refer to spectra measured in deuteriochloroform) are characteristic of the allocyathin B_3 series. The observed coupling constant of 5.5 Hz is typical of olefinic coupling in a five-membered ring[39]. The observed chemical shifts indicated the presence of a cyclopentadiene rather than a cyclopentene moiety (ref 35b, p 188: δ 6.42 and δ 5.60).

The signal pattern in the region δ3.0-2.0 is simplified by the absence of signal (h, i). Signal j is no longer hidden under the acetyl peaks u and v and an eight-line pattern with large couplings is readily analyzable. Likewise, the coupling constants of signals f and g are obtained without difficulty.

Absorption in the region δ 1.9-1.1 integrated for four protons, two less than in the case of O,O-diacetylcyathin A_3. Since all other protons interact only among themselves and since the signal pattern in the region

δ 1.9-1.1 is very complex (no singlets and no AB quartets), it can be concluded that the pattern constitutes an isolated ABCD-system. The large geminal and/or vicinal couplings indicate that the corresponding protons are attached to adjacent carbon atoms.

The essential difference between the cyathin A_3 and allocyathin B_3 series is that protons h, i, k and l have been replaced by protons y and z, i.e. a $-CH_2 - CH_2-$ replaced by a -CH=CH- grouping. The nmr data suggest the presence of a cyclopentadiene structure. The double bond revealed by the signals y and z must therefore be conjugated to another double bond. As these signals show no coupling other than with themselves, the second double bond must be tetrasubstituted. Likewise, the saturated carbon of the cyclopentadiene must be fully substituted. This leads to part structure 32 for allocyathin B_3 and consequently to 33 for cyathin A_3. The shift of protons h and i agrees closely with that of the allylic protons in cyclopentene: δ 2.3 versus 2.28 (ref 35b, p 188).



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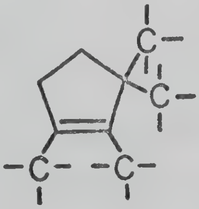


33

The uv spectrum (in methanol) of the acetyl derivative is more structured than that of the parent compound. It has a maximum at 229 nm (ε = 16,000) and a shoulder at 255 nm (ε = 9,100). The observed curve above 300 nm is plain, but a hidden absorption band is possible in this region, since the extinction coefficient at 320 nm, for example, is 170. The observed curve can be interpreted in terms of two overlapping chromophores: an α,β-unsaturated ketone and a cyclopentadiene. Upon ketalization, the ketone chromophore should be removed and the diene chromophore should give rise to an undisturbed absorption.

Ketalization of allocyathin B_3 was carried out under the same conditions used in the ketalization of cyathin A_3. Again, the reaction was followed by monitoring the dichroic absorption at 326 nm. In two experiments the following reaction rates were observed:

	$t_{50\%}$	$t_{90\%}$
run 1	4 min	120 min
run 2	8 min	40 min

The initial rates are similar to those observed for cyathin A_3; however, it takes much longer for the reaction to go to completion. The crude product requires

chromatographic purification, and even then some impurities seem to persist. It thus appears that some further reaction(s) occurs after initial ketalization. In a third experiment the reaction time was reduced to 30 min, which seemed to improve the situation, although the nmr spectrum (see below) still showed the presence of some impurities in the apparently (by tlc) pure ketal. Epimerization at the carbon bound to proton b or acid catalyzed isomerization of the cyclopentadiene moiety might be responsible for the side reactions. Alternatively, impure starting material may be responsible for the difficulties, as noncrystalline allocyathin B_3 was used.

The molecular weight and composition of allocyathin B_3 methyl ketal were determined by mass spectrometry (Figure 37): mw 330; $C_{21}H_{30}O_3$. The base peak is again at m/e 141, due to a fragment $C_7H_9O_3$. In general, the mass spectrum is similar to that of the ketal of cyathin A_3, except that the peaks are shifted by two mass units to lower mass.

The ir spectrum (Figure 38) shows some remaining hydroxyl absorption and the absence of carbonyl absorption. Two barely perceptible bands at 1650 and 1620 cm^{-1} indicate C,C-double bonds. Two weak but sharp bands at 3100 and 3050 cm^{-1} (olefinic C-H stretching) most clearly

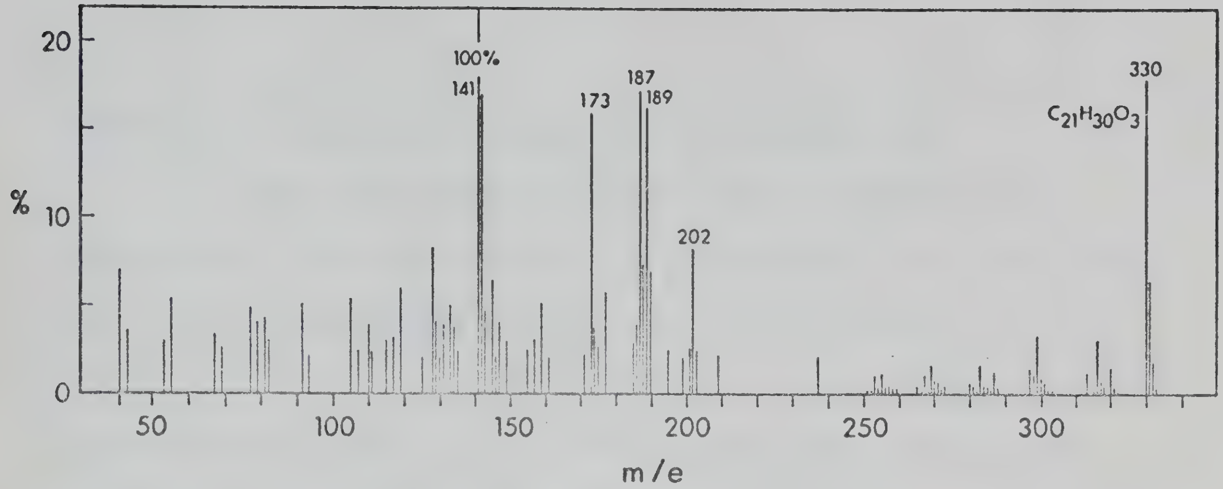


Figure 37. Mass spectrum of allocyathin B_3 methyl ketal

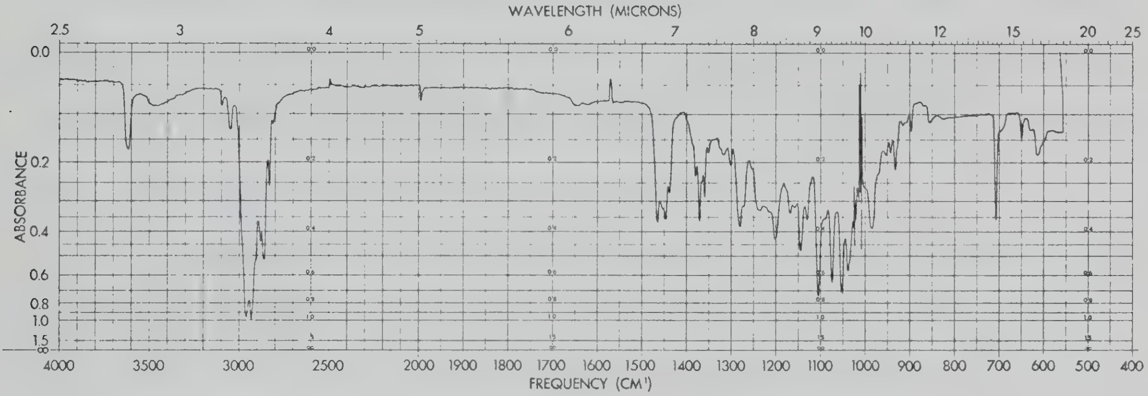


Figure 38. Ir spectrum of allocyathin B_3 methyl ketal (CCl_4)

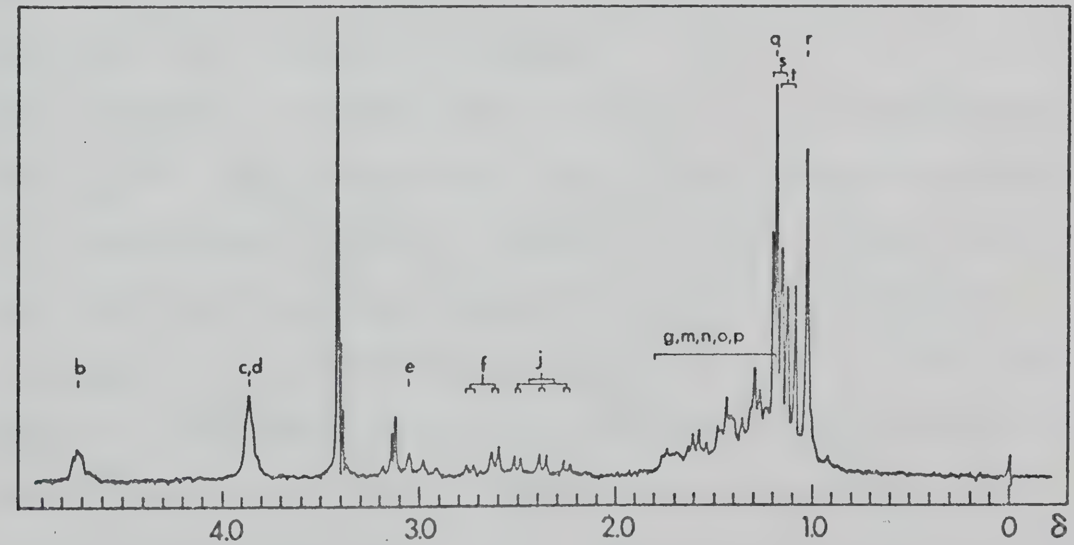


Figure 39. 100 MHz nmr spectrum of allocyathin B_3 methyl ketal (C_6D_6)

distinguish this ketal from that of cyathin A_3.

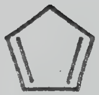
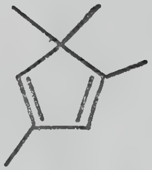
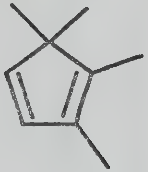
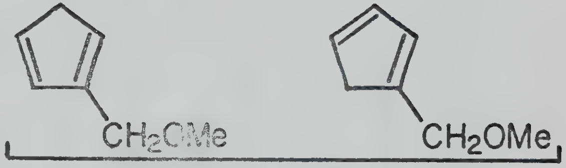
The nmr spectrum (Figure 39) indicates the presence of some impurities. Nevertheless, the spectrum can still be analyzed without difficulty (Table V). The signal pattern is by now familiar: signals f and j (unobstructed by signal (h, i) and slightly more separated than the corresponding signals in the ketal of cyathin A_3) are now easy to interpret. Signal g is again lost in the signal heap between δ 1.8-1.1. It should be kept in mind, however, that the assignments for f and g are tentative and that they might have to be reversed.

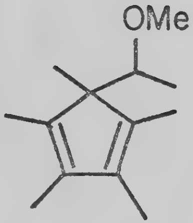
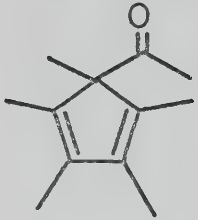
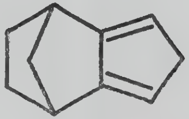
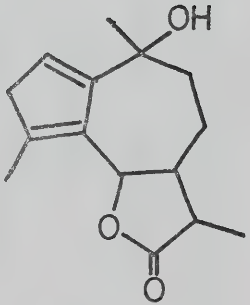
The uv spectrum drastically changed by ketalization: in isooctane, a maximum is observed at 256 nm with $\varepsilon = 4{,}200$; the extinction at 320 nm has dropped to $\varepsilon = 20$. According to the Woodward-Fieser rules for conjugated polyenes, neither a heteroannular nor a homoannular diene would have an absorption maximum at 256 nm, if the realistic assumption is made that the diene is tetrasubstituted ($214 + 20 = 234$ nm and $253 + 20 = 273$ nm being the respective calculated values). However, it must not be forgotten that the rules were established for double bonds incorporated in six-membered rings. As can be seen from Table VI, the rules do not apply at all to cyclopentadienes. The uv data for the ketal agree quite well with those of the tetrasubstituted compounds listed in Table VI.

Table V. Nmr Data of Allocyathin B_3 Methyl Ketal and O-Acetylcyathin B_3 Methyl Ketal

Signal	Shift in $CDCl_3$ (δ)	Shift in C_6D_6 (δ)	Multiplicity	Couplings with Signals	Coupling Constants (Hz)	Shift of Acetyl Derivative in C_6D_6 (δ)
a	6.11	-	dd	c,d	1.5,1.5	5.93
b	4.82	4.72	u	(j)	(3)	4.55
c	4.35	3.86	u	(d,a)	(14,1.5)	4.44
d	4.35	3.86	u	(c,a)	(14,1.5)	4.30
e	3.00	3.05	qq	s,t	7.7	2.95
f	?	2.68	dd	j,g	12.5,3.5	2.60
g	?	?	(dd)	(j,f)	(12.5,3.5)	?
j	?	2.48	d,d,d	f,g,b	12.5,12.5,3	2.30
m,n,o,p	1.8 - 1.1	1.8 - 1.1	m	?	?	1.8 - 1.1
q	0.97	1.18	s	-	-	1.14
r	0.83	1.02	s	-	-	1.03
s	1.13	1.16	d	e	7	1.12
t	1.07	1.12	d	e	7	1.08
u	-	-	-	-	-	1.59
w (OCH_3)	3.36	3.41	s	-	-	3.27
y	6.30	-	d	z	5.5	6.36
z	6.22	-	d	y	5.5	6.23

Table VI. Uv Data of Cyclopentadienes

				
λ_{max}	240	256 or 257	256 or 257	247
ε_{max}	2,500			4,000
Solvent	heptane	methanol	methanol	cyclohexane
Reference	(40)	(41)	(41)	(42)

					
λ_{max}	252	253	243	i) 248	ii) 248
ε_{max}		3,300	4,000	4,500	3,100
Solvent	methanol	isooctane		ethanol	
Reference	(43)	(44)	(45)	(46)	(47)

Thus the part structure 32 which was arrived at from nmr data is strongly supported by the uv spectrum.

The conjugated diene chromophore is also revealed by the chiroptical methods. A negative Cotton effect centered at 256 nm is superimposed on a plain negative ord curve; the cd curve has a negative minimum at 251 nm with a dichroic absorption of $\Delta\varepsilon = -3.6$. The Cotton effect in both cases is undisturbed by other chromophores.

The ketal was acetylated under very mild conditions (dilute methylene chloride solution, small amount of pyridine, sixfold excess of acetic anhydride, 1 hour at 30°, 1/2 hour at 40°). Since impure ketal was used as starting material, impure product was obtained, which was then purified by ptlc. The chromatographically pure sample still contained some minor impurities according to nmr spectroscopy.

Mass spectroscopy (Figure 40) established that a monoacetate had formed, since the molecular weight was 372 and the molecular formula $C_{32}H_{32}O_4$. Acetylation caused the base peak to shift from m/e 141 to 183.

The ir spectrum displayed the weak bands at 3100 and 3050 cm^{-1}; hydroxyl absorption at 3620 and 3470 cm^{-1} had vanished; carbonyl absorption appeared at 1745 cm^{-1}.

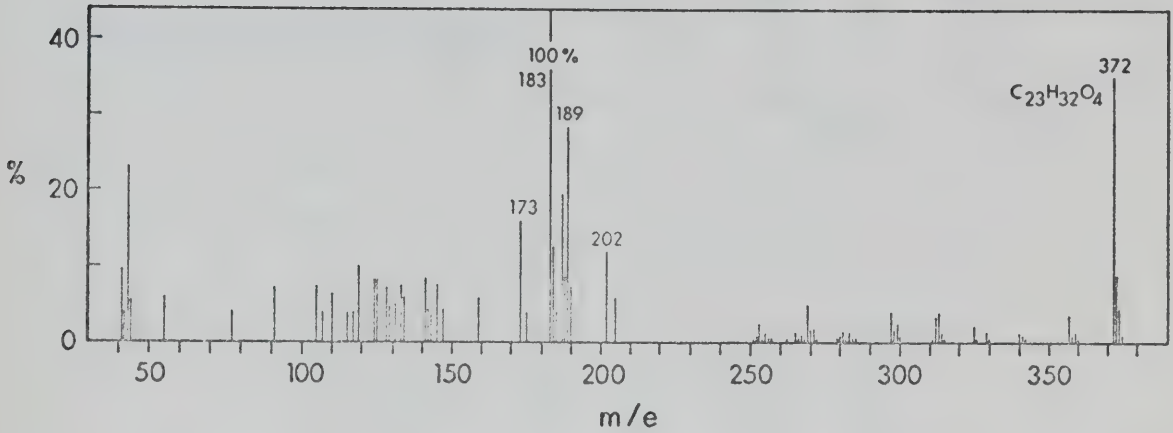
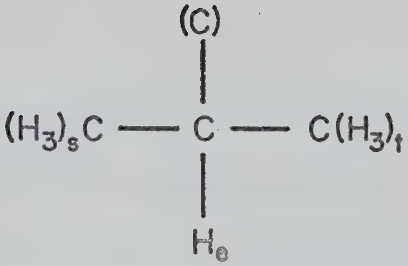


Figure 40. Mass spectrum of O-acetylallocyathin B_3 methyl ketal

The nmr data are compiled in Table V. A single peak at δ 1.59 (in C_6D_6) confirms monoacetylation; the methylene protons c and d of the primary hydroxyl group have undergone a downfield shift of 0.49 ppm, typical for acetylation. No other pertinent changes occurred.

The optical and chiroptical properties remained unaffected as would be expected.

The various part structures revealed by spectroscopic investigations are listed below. Part structure 22 is likely not contained in a six-membered ring.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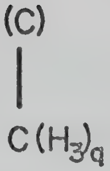
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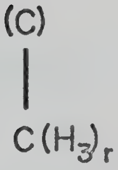
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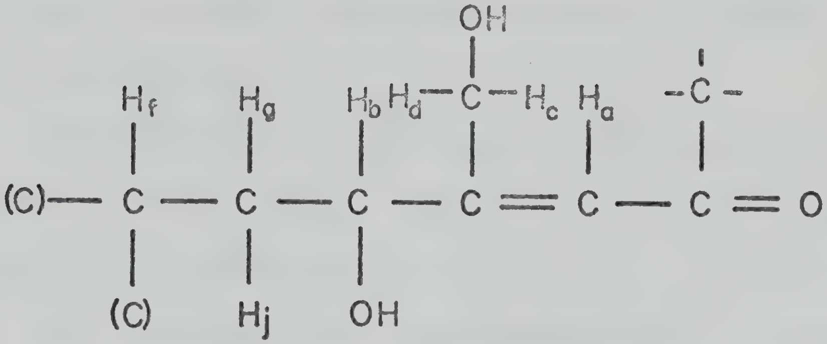
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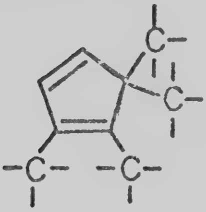
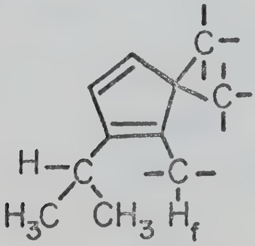

32

The low chemical shifts of protons e and f suggest that the corresponding carbon atoms are attached to the cyclopentadiene moiety 32. This leads to the alternative part structures 34 and 35.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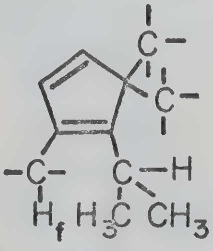
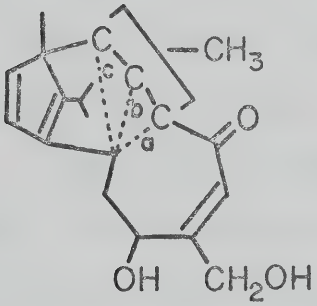

35

Twenty-four of the 28 protons and 17 of the 20 carbon atoms are accounted for in these part structures. At least two of the remaining four protons are strongly coupled with each other. Since the number of double bonds is four and the unsaturation number is seven, the molecule is tricyclic.

The remaining three-carbon part structure must be attached to the carbonyl carbon, to the carbon bearing proton f, and, possibly, to the C-5 of the cyclopentadiene. The only ways in which this can be achieved are depicted in structures 36, 37, 38 and 39.



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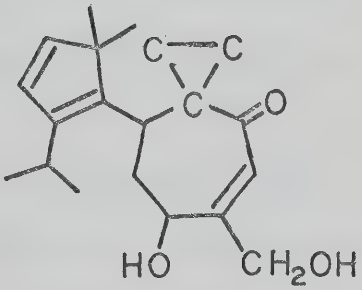

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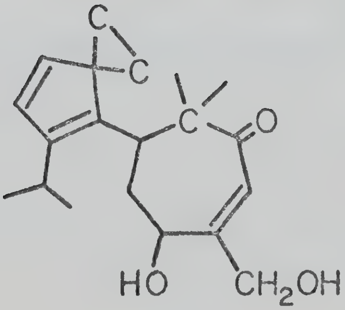

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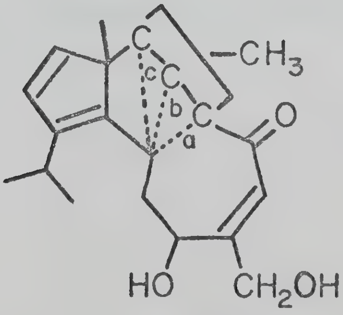


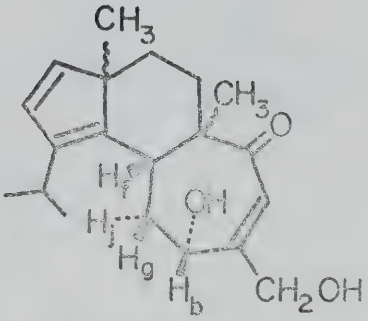
39

Structures of type **36** are ruled out since they contradict Bredt's rule, i.e. they are extremely strained. Structure **37** (or its double bond isomer) can likewise be disregarded because the nmr spectra lack signals in the region typical of cyclopropyl protons, i.e. below δ 1.0 (upfield from δ 1.0). Moreover, a structure such as **37** could not account for the ion $C_{14}H_{21}$ at m/e 189, formed as the main fragment on electron impact. Structure **38** can be excluded for the same reasons.

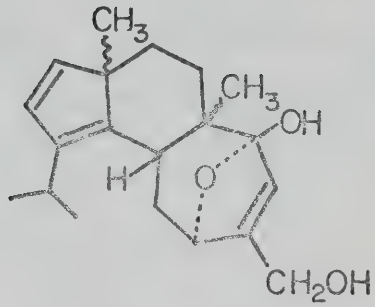
Structure **39c** has a methylene group in the α-position of a keto group. The corresponding protons should give rise to signals above δ 2.0 (i.e. downfield from δ 2.0). Since this is not observed, this structure can also be ruled out.

The same reasoning applies to structure **39b**. Moreover, it contains two isolated methylene groups which should give rise to two discernible AB quartets. Again this is not observed.

Finally, then, **40** (=**39a**) remains as the unique structure for allocyathin B_3 which accomodates all of the evidence.



40

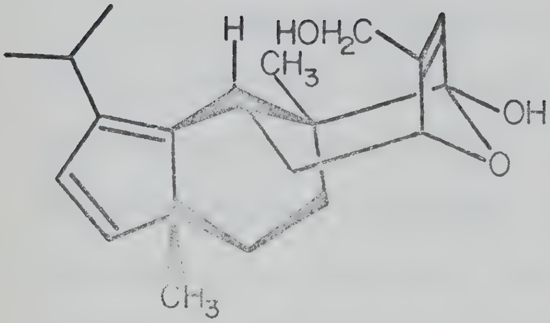


41

Some relative stereochemical assignments, again based on nmr data, are included in structure 40. Proton f is arbitrarily selected to be on the β-face. The large coupling constant between proton f and proton j suggests that these two have a *trans* diaxial relationship. The coupling pattern observed for the ketal derivatives reveals a "pseudo-*exo*" configuration for proton j. It follows then that proton b is *trans* related to proton j. Consequently, the ether bridge in the hemiketal form 41 is located on the α-side.

It follows from consideration of models that if ring B (see structure 44 for lettering) is in a chair conformation, then the two angular methyl groups must be *trans* to one another, as in structure 42 (*cis* B/C ring juncture) or 43 (*trans* B/C ring juncture).

On the basis of the evidence presented it is not possible to decide which represents the stereochemistry,



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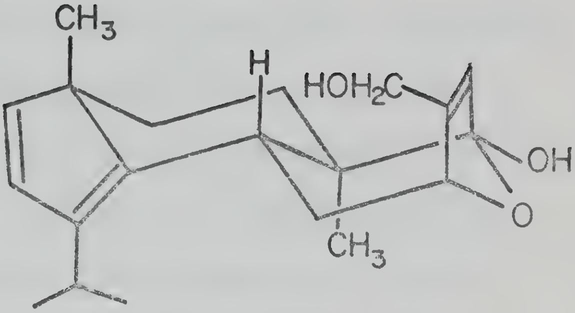
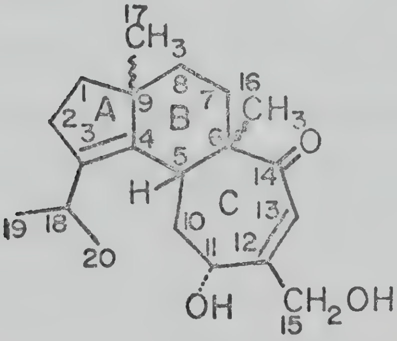


43

or indeed, since we cannot rule out the possibility that ring B is in the twist conformation, to draw any further firm stereochemical conclusions.

Since the many spectroscopic measurements suggest that cyathin A_3 is dihydroallocyathin B_3, structures 44 and 45 are proposed for the ketone and hemiketal form of cyathin A_3, respectively. The numbering of the carbon atoms and the lettering of the rings adopted for further discussions is indicated in structure 44.



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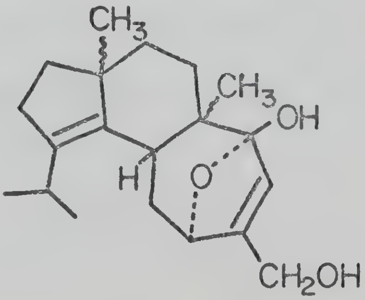
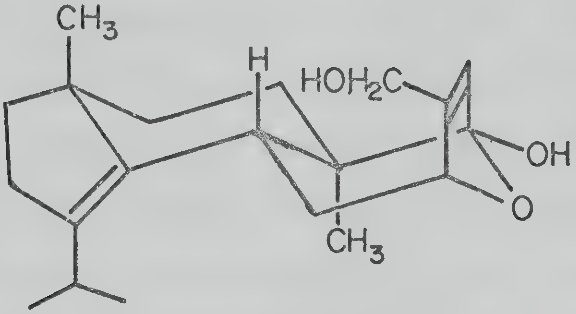


45

The constitution of cyathin A_3 has been confirmed by an X-ray crystallographic investigation, carried out by Drs. M.J. Bennett and R.M. Tuggle[48]. The X-ray analysis (see Appendix 2) confirms that cyathin A_3 crystallizes in the hemiketal form and also reveals the complete relative stereochemistry. The conformation in the solid state can be approximated by structure 46, in which both six-membered rings assume chair forms.



46

The absolute stereochemistry remains to be determined.

Up to this point the assumption has been made that allocyathin B_3 is 1,2-dehydrocyathin A_3, and evidence from both series has been used to solve the structural problem. It was now necessary to justify this assumption by correlating the two series.

4) Correlation of Cyathin A_3 and Allocyathin B_3

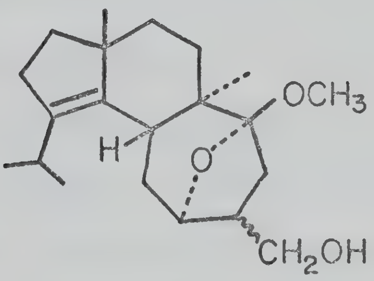
Hydrogenation of cyathin A_3 or dehydrogenation of allocyathin B_3 should be the simplest reactions to correlate the two compounds. A controlled dehydrogenation would probably require a multi-step procedure (e.g. oxidation or halogenation at C-2, followed by dehydration or dehydrohalogenation) and has not been attempted. It was anticipated that hydrogenation would also be accompanied by difficulties, as allocyathin B_3 has four sites of unsaturation which could become involved in a hydrogenation reaction: reduction of the carbonyl group could, potentially, lead to one pair of epimers, saturation of the trisubstituted double bond to another pair, and saturation of the tetrasubstituted double bond to another two pairs. Considering further that side reactions such as isomerization and hydrogenolyses could occur, the number of potential products becomes large.

A smoother hydrogenation reaction might result by using the methyl ketal as starting material: one of the unsaturated sites, the carbonyl group, is masked; the chances for stereospecific hydrogenation are increased, since the ketal has a more rigid conformation; in general, the ketal appears to be more stable and, therefore, should lead to fewer side products.

Hydrogenation of conjugated dienes is believed

to follow predominantly a 1,2-addition mechanism[49]. Tetrasubstituted double bonds require more drastic reaction conditions than less substituted double bonds in order to undergo hydrogenation. It therefore appeared possible to saturate the disubstituted double bond of the cyclopentadiene moiety selectively by carrying out the reaction at atmospheric pressure and room temperature.

However, it was felt that the Δ^{12} double bond might also be affected under hydrogenation conditions. It was, therefore, decided to carry out the reaction first with cyathin A_3 methyl ketal. In a few exploratory experiments it was soon seen that a number of products are formed. The principal products appeared to be **47**, **48**, **49** and **50**.



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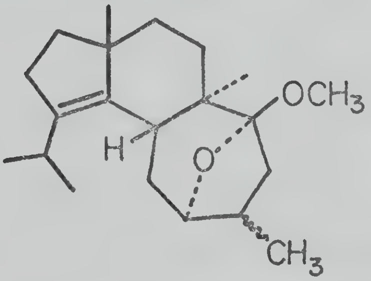

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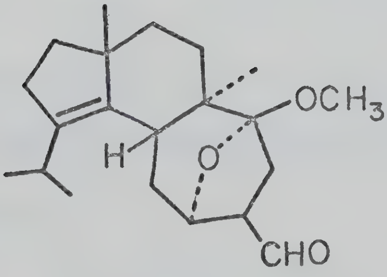

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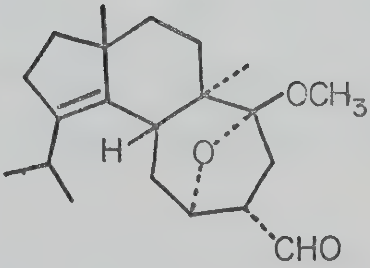


50

Compounds **47** and **48** and the epimers **49** and **50** give rise to well-separated spots and can be isolated without difficulty by ptlc. The polarity (R_f-value) of the compounds decreases (increases) as expected in the order alcohol, aldehyde, "hydrocarbon". It is possible that the materials assigned structures **47** and **48** each consist of a mixture of C-12 epimers, since both were characterized by ir and mass spectrometry only (**47**: mw 334, $C_{21}H_{34}O_3$, hydroxyl absorption; **48**: mw 318, $C_{21}H_{34}O_2$, no hydroxyl absorption).

Compound **50** readily crystallized: crystals were deposited from both ether and carbon tetrachloride solutions when the solvent was allowed to evaporate. Hydrocarbon solvents such as isooctane or Skellysolve B appeared to be the most suitable for recrystallization. The melting behavior as observed on a hot-stage melting-point apparatus appears anomalous: the crystals transform into a slush at 113-115° and do not give a clear melt even at 135°. Molecular weight and composition were determined by mass spectrometry (Figure 41): 332, $C_{21}H_{32}O_3$. Compound **50** is an isomer of the starting material. The fact that the peak at m/e 141 is no longer of great intensity, however, leaves no doubt that **50** is different from the starting material.

The solid-phase ir spectrum (Figure 42) reveals

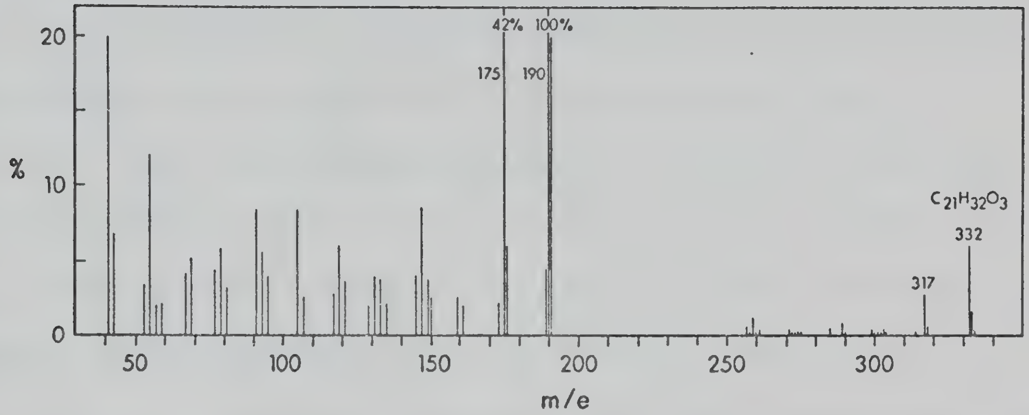


Figure 41. Mass spectrum of aldehyde 50

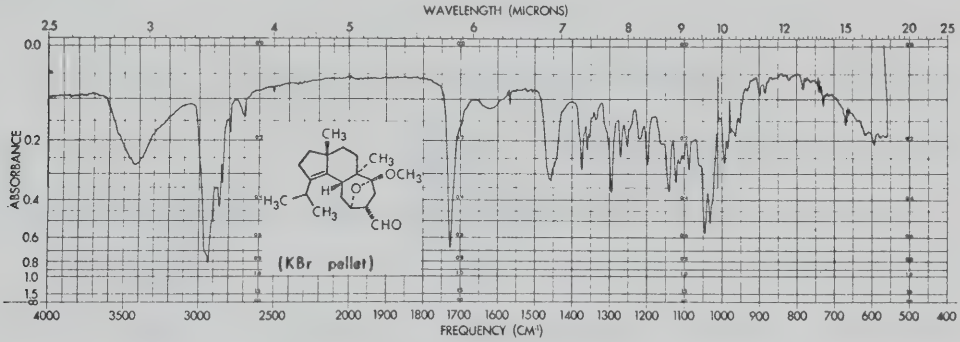


Figure 42. Ir spectrum of aldehyde 50 (KBr pellet)

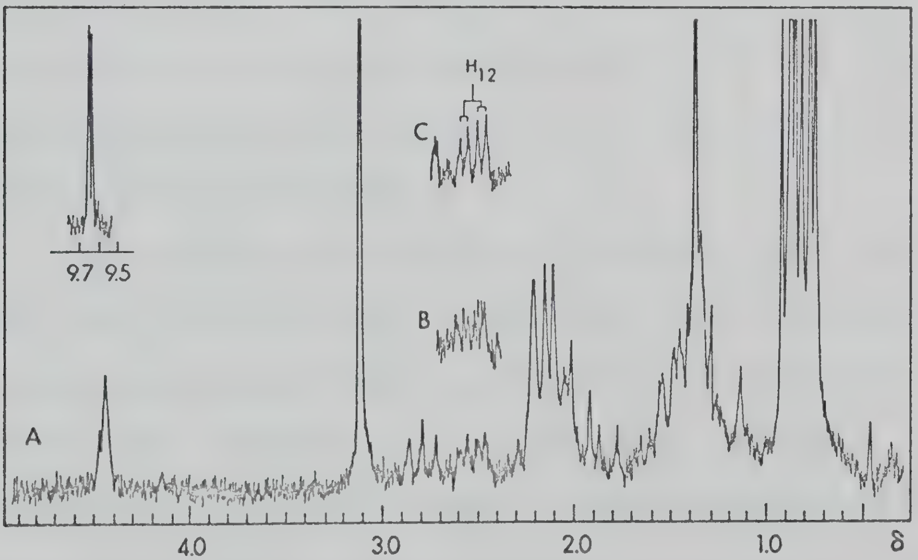


Figure 43. 100 MHz nmr spectrum of aldehyde 50 (CCl_4); A, undecoupled; B, signal at δ 4.45 irradiated; C, signal at δ 9.64 irradiated

absorption characteristic of an aldehyde, at 2710 and 1725 cm^{-1}. The hydroxyl absorption must be due to water, since it is absent in the solution spectrum (Figure 44).

The nmr spectrum (Figure 43) shows a characteristic aldehyde signal at δ 9.64 with $J_{12,15}$ = 2 Hz. Proton H_{12} gives rise to a complex signal at δ 2.53. Its coupling pattern can be detected only by double irradiation: $J_{12,13a}$ = 9, $J_{12,13b}$ = 4, $J_{12,15}$ = 2 Hz. The nmdr experiment also shows clearly that there is, at most, a very small coupling ($J<0.5$ Hz) of H_{12} with H_{11}. Consequently, H_{12} occupies the endo position (i.e. it is trans to the oxygen bridge) and the aldehyde function the exo position[50].

Compound 49 is slightly more polar than 50. If allowed to stand in methanol containing sodium carbonate, it isomerizes to 50. The reaction can be followed by tlc and the identity was established by tlc and ir spectroscopy. As C-12 is the only epimerizable center, it follows that 49 is the C-12 epimer of 50.

The product distribution depends on the type of catalyst and the pH of the solution used for hydrogenation. (Other factors such as solvent, addition of promoters and inhibitors, and temperature were not investigated.) Platinum and rhodium catalysts seemed to favor production of the alcohol(s). Much hydrogenolysis occurred under acidic conditions, whereas in a strongly basic medium, hydrogena-

tion seemed to be inhibited altogether. It appeared that the best results were obtained in neutral or slightly alkaline solution. Neutral conditions led to aldehyde 49, and slightly basic conditions to aldehyde 50, as well as to other products.

Some of the above observations were actually made using allocyathin B_3 methyl ketal, but have been included here in order to provide a more concise description.

Since aldehyde 50 readily crystallized and its spectra displayed several distinctive features, it was decided to correlate cyathin A_3 and allocyathin B_3 via this compound. When allocyathin B_3 methyl ketal was subjected to hydrogenation, using neutral conditions, aldehyde 49 was obtained. When 49 was then subjected to equilibration in methanol containing sodium carbonate, 50 was produced, as shown by tlc and ir spectroscopy (Figure 44). Despite the fact that the aldehyde from allocyathin B_3 could not be obtained crystalline (the experiments were conducted on a very small scale), the close identity of the ir spectra does support the validity of the correlation.

The hydrogenation experiments finally confirm earlier chemical and spectroscopic evidence that cyathin A_3 has structure 44. Since the relative stereochemistry of

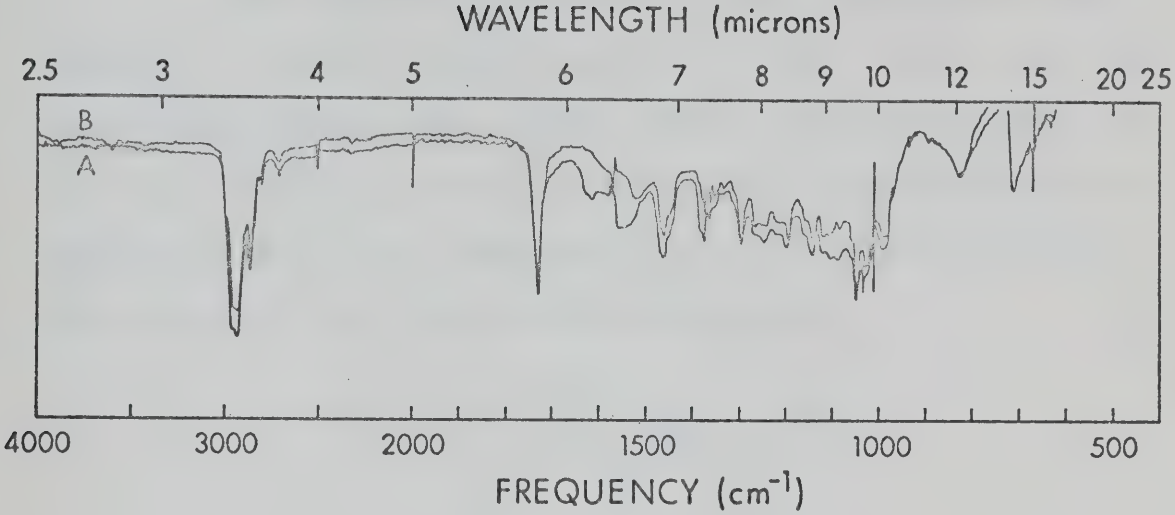
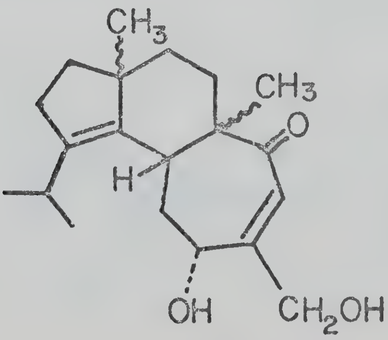


Figure 44. Comparison of the ir spectra of aldehyde 50 obtained from cyathin A_3 methyl ketal (B) and allocyathin B_3 methyl ketal (A) (CCl_4)

cyathin A_3 was revealed by X-ray analysis, these same experiments also establish the stereochemistry of allocyathin B_3, as shown in 51. None of the asymmetric centers C-5, C-6, C-9 and C-11 should be affected during hydrogenation.



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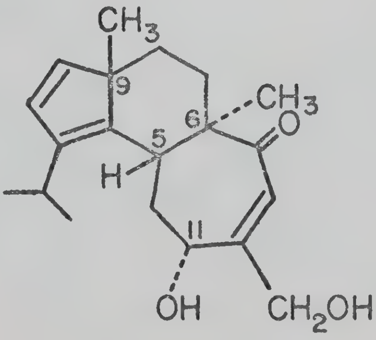
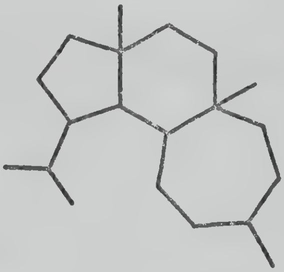


51

5) Biogenetic Considerations

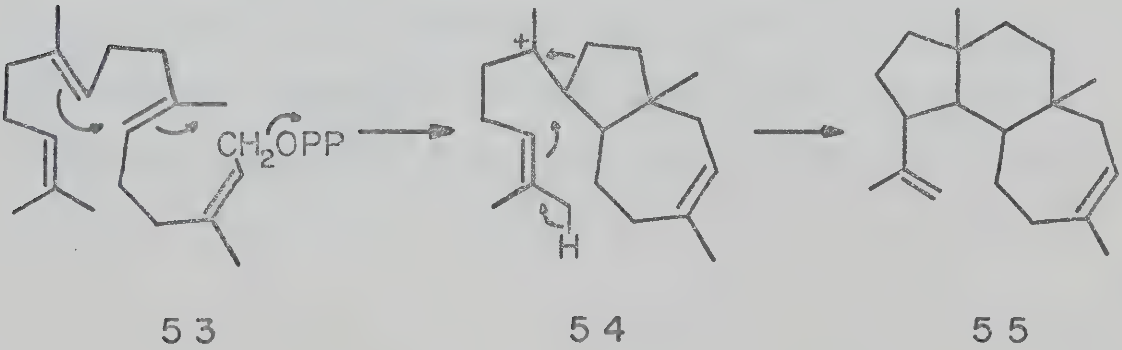
The molecular formulas of the cyathins and the number of C-methyl groups present in them suggest that they are diterpenoids. The carbon skeleton 52 established for the cyathins is not among those listed in the "Handbook of Naturally Occurring Compounds"[51] and to the best of our knowledge has not been reported elsewhere.



52

Geranylgeranyl pyrophosphate (53) is regarded as the natural precursor of the diterpenoids[52]. The carbon skeleton of the cyathins may be derived according to the hypothetical Scheme IV outlined below. Oxidation and reduction at the various centers to the appropriate levels at some stage of the biosynthesis will lead to the various individual cyathin components.

Scheme IV



III. GENERAL EXPERIMENTAL

Purity of Solvents and Adsorbents

Commonly used solvents (reagent grade) were checked for purity. The following amounts of residue were obtained after distillation at atmospheric pressure.

Solvent	Residue [mg/l]
acetone	15
benzene	10
chloroform	10
ethyl acetate	30
methanol	7

The residues seemed to consist mainly of rust and silicone grease. However, acetone and ethyl acetate contained impurities that had R_f-values similar to those of cyathin constituents. For many purposes the amount of contamination was intolerable; the solvents were therefore distilled prior to use.

The purity of adsorbents was checked in the following way: 20 g adsorbent was stirred in 200 ml solvent, the adsorbent removed by careful filtration, the solvent evaporated under reduced pressure and the residue weighed.

Adsorbent	Solvent	Residue [mg/20 g]
silicic acid (Mallinckrodt, 100 mesh)	chloroform	1.1
silica gel G (E. Merck)	acetone	2.6

No convenient method was found to remove the contaminants and the adsorbents were used directly.

Thin-Layer Chromatography

1) Preparation of Thin-Layer Plates

Thin-layer plates were prepared according to the method described in Stahl's handbook[20], p 27, using DESAGA equipment.

The two principal adsorbents used were: silica gel G (E. Merck) containing 1% electronic phosphor ($ZnSiO_4$, General Electric) referred to as ordinary silica gel; and silica gel G impregnated with 10% silver nitrate (argentated silica gel). In order to obtain an adsorbent layer of 0.05 x 20 x 100 cm, slurries made of 50 g silica gel and 110 ml H_2O or of 45 g silica gel and 100 ml of an aqueous solution containing 5 g $AgNO_3$, were prepared. The adsorbent layers were activated at 120° for 2 hr. In the case of argentated silica gel, particular care was taken to use clean materials and equipment and a dust-free oven; otherwise discolored layers resulted that made subsequent detection difficult or impossible. Layers were protected

from light as much as possible and preferably were used the same day. The tlc spreader was cleaned immediately after use, since the argentated adsorbent is very corrosive.

The dimensions of the plates used for preparative work were 20 x 100 or 20 x 20 cm; for analytical work, 20 x 20, 20 x 10 or 20 x 5 cm. The thickness of the layer for preparative work was 0.5 - 0.7 mm; for analytical work, 0.3 - 0.5 mm. For certain purposes (following the course of a reaction or the progress of a chromatographic separation), microslides (1 x 3 in. and 2 x 3 in.) coated with ordinary silica gel were used. These were prepared by dipping the slides into a suspension of the adsorbent in chloroform.

2) Application of Samples

In analytical work, the sample was applied with the aid of a capillary. A manual application method was also used for preparative work. The sample was dissolved in an amount of solvent such that the viscosity of the solution was low enough to allow free flow through the orifice of a micropipet (200 mg/1 ml was satisfactory in most cases). The least polar solvent possible (and preferably with a boiling point of 50-80°) was chosen for dissolving the sample. Skellysolve B (a hydrocarbon mixture with a boiling point of ∿65°) was the ideal choice, but because of solubility problems carbon tetra-

chloride, chloroform or acetone frequently were substituted. It was necessary to filter the solution through a plug of cotton wool to remove lint, etc. Micropipets holding a volume of $\sim$0.5 ml and having an orifice of <0.1 mm were manufactured from ordinary glass tubing (internal diameter 4 mm), and fitted with a soft rubber bulb. The pipets were tested before use with pure solvent and only those which gave a very fine, uninterrupted jet of solvent were used to apply the solution to the tlc plate. A 300-mg sample in 1 ml solvent could be applied in a straight line by moving the pipet approximately five times across a 20 x 100 cm plate. The technique required a lot of practice at first (particularly with regard to "straight line" and "uninterrupted jet"), but subsequently was used very efficiently.

For initial, rough separations 200 - 300 mg of substance were used on a 20 x 100 cm plate; for finer separations, 100 - 200 mg were used.

3) Detection of Spots or Bands

In analytical work with ordinary silica gel, spots were visualized by spraying the plates with 30% H_2SO_4 and subsequent heating them to $\sim$110° (or vice versa), or were detected by viewing under short-wave uv-light (254 nm). The latter method is very sensitive for compounds containing chromophores that absorb in the 250 nm

region, e.g. conjugated enones and dienes.

The uv method was also used extensively for preparative purposes. Alternatively, particularly in cases of nonabsorbing compounds, chromatography on an analytical tlc plate was carried out first. The spots were detected by the H_2SO_4-method and the results extrapolated to preparative separations.

The H_2SO_4-method was also applicable for analytical purposes on argentated silica gel. A non-destructive detection method involved spraying the plates with a 0.2% solution of 2',7'-dichlorofluoresein in ethanol followed by viewing under long-wave uv-light (325 nm).

4) Recovery of Material After Chromatographic Separation

Zones containing organic material were marked with a pointed glass rod. The corresponding absorbent was removed from the glass plate with the aid of a microslide. (Microslides proved superior to any other device.) The material was then placed in a chromatographic column of such dimensions that an adsorbent layer of about 10 cm height resulted. The organic material was slowly eluted with ether. (Acetone was used in the earlier part of this work, but later on ether proved polar enough to allow efficient elution of all compounds encountered in this work.)

5) Recording of R_f-Values

R_f-values are recorded in the form $R_f(X)n$. The letter X refers to the solvent system used for developing; the number n is obtained by dividing the distance travelled by the particular component by the distance travelled by the solvent front. In cases in which argentated silica gel was used, the expression "$AgNO_3$" is added within the parenthesis.

It should be noted that the tlc experiments were not carried out under standardized conditions, i.e. age of plates, tank saturation, etc., varied. Consequently, the R_f-values are not strictly reproducible and it is estimated that they might vary by 0.1 - 0.2 units.

Abbreviations for Solvent Systems

The following abbreviations for solvent systems have been used throughout this thesis:

A:	benzene-dioxane-acetic acid	100:25:1
B:	benzene-acetone-acetic acid	70:30:1
C:	benzene-acetone-acetic acid	75:25:1
D:	benzene-acetone-acetic acid	80:20:1
E:	alcohol free chloroform	
F:	chloroform (0.75% ethanol)	
G:	chloroform-methanol	98:2
H:	chloroform-methanol	95:5
I:	chloroform-methanol	90:10

J:	Skellysolve B-acetone	9:1
K:	Skellysolve B-acetone	7:1
L:	Skellysolve B-acetone	5:1
M:	Skellysolve B-acetone	3:1
N:	Skellysolve B-acetone	2:3

Sublimation

The term "sublimation" has been loosely used. It includes evaporation and condensation under reduced pressure (oil pump) of gum-like substances. The terms "molecular distillation", "evaporative distillation", "substillation" have been used elsewhere for the same process.

Acetylation Procedures

Procedure 1) A mixture of 20 mg substrate, 1 ml pyridine and 0.5 ml acetic anhydride was allowed to stand at room temperature overnight.

Procedure 2) A mixture of 20 mg substrate, 1 ml pyridine and 0.5 ml acetic anhydride was allowed to stand at room temperature for 2 hr.

Procedure 3) A mixture of 20 mg substrate, 0.16 ml pyridine, 0.08 ml acetic anhydride and 1 ml methylene chloride was allowed to stand at room temperature for 1 hr and then at slightly elevated temperature (∿40°) until the reaction was complete (as indicated by tlc).

The reaction mixture (after using any of the procedures) was taken up in benzene (∿5 ml) and evaporated under reduced pressure to remove the bulk of the reagents. The residue was taken up in benzene (∿2 ml) and washed with one drop of water. The benzene layer was removed and evaporated to afford the crude acetyl derivative.

Alternatively, the bulk of the reagent was removed by evaporation (30°/1 - 2 mm), the residue taken up in ether (∿20 ml), washed with small amounts of water, dried ($MgSO_4$) and evaporated to afford the crude acetyl derivative.

Measurement and Recording of Spectra

All mass spectra reported in this thesis were measured on an A.E.I. model MS-9 mass spectrometer and are recorded as a percentage of the most intense peak (base peak). High resolution mass spectrometry (hrms) was used in most cases to determine molecular formulas. The measurements were carried out by using a direct probe (70 - 200°) at an ionization energy of 70 eV.

Infrared (ir) spectra were recorded on a Perkin-Elmer model 421 dual grating or a Unicam SP 1000 infrared spectrophotometer.

The Raman spectrum of solid cyathin A_3 was recorded on a Laser-Raman instrument manufactured by Spex Industries Inc.

The 220 MHz nuclear magnetic resonance (nmr) spectra were measured on the 220 MHz Proton Nuclear Magnetic Resonance Spectrometer at the Ontario Research Foundation, Sheridan Park, Ontario. The other nmr spectra were measured on a Varian Associates model A-60 or HR-100 spectrometer. Nuclear magnetic double resonance (nmdr) experiments were also performed with the latter instrument.

Tetramethylsilane (tms) was used as an internal standard for samples measured on the 60 and 220 MHz instruments. Tms also provided the internal lock signal for most of the experiments carried out with the HR-100 instrument. In some instances, chloroform or benzene was used for this purpose and tms was then added to produce the reference signal. However, in some experiments in which benzene-d_6 was used as solvent and benzene-h_6 as the lock signal, the addition of tms was omitted; in these cases the tms signal was assumed to be at 7.20 ppm upfield from the lock signal. The δ-scale has been used throughout this thesis to express chemical shift values. It is estimated that the δ-values might have an absolute error of ±0.1 ppm. (Differences of this order were regularly encountered when the measurement of a particular sample was repeated.) However, the relative error (important for shift separations and coupling constants) should be much less (∿±0.005). The pattern of signals is denoted by: s = singlet, d = doublet, t = triplet, q = quadruplet,

m = unresolved multiplet, b = broad, u = unresolved band.

Most of the ultraviolet (uv) spectra were recorded on a Cary Recording Spectrophotometer, model 14M. Optical rotatory dispersion (ord), circular dichroism (cd), some uv spectra and optical rotations ($[\alpha]_D$) were determined on a Durrum-Jasco Recording Spectropolarimeter. The determination of the wavelengths of the maxima is probably very accurate (error less than 1 nm); however, it is estimated that the values of the extinction coefficient (ε), molecular rotation ($[\phi]$), and dichroic absorption ($\Delta\varepsilon$) could have an error of as much as 50% since quantities of the order of 2 mg were measured on a balance that had an estimated accuracy of ±0.5 mg.

Sample Preparation for Spectroscopic Measurements

It was difficult to obtain solvent-free samples in the case of gum-like substances. Even after prolonged drying at ∿1 mm some traces of solvent were usually still present.

When the spectrum of a substance was to be measured in solution, "purging" with the appropriate solvent proved very effective in overcoming this difficulty. The sample was dissolved in a small amount of the solvent to be used and the solvent was subsequently removed by evaporation under reduced pressure; this process was then repeated twice. All volatile contaminants could be removed

in this fashion. Insoluble impurities were then removed by filtration through a plug of cotton wool.

Samples were submitted to the various Spectroscopic Services of this Department for the measurement of mass, ir and nmr spectra (except 220 MHz nmr spectra); some of the ir and most of the uv, ord and cd spectra were measured by the author.

Melting-point Apparatus

In the few instances that crystalline compounds were obtained, the melting points were determined on a Leitz-Wetzlar hot-stage melting-point apparatus fitted with a microscope. Alternatively, a Fisher-Johns melting-point apparatus was used to determine the melting point of chromocyathin and its derivatives.

Microanalyses

Microanalyses of chromocyathin and its derivatives were performed by the Microanalytical Laboratory of this Department.

IV DETAILED EXPERIMENTAL

Production and Isolation of Crude Cyathin

(Taken from B. N. Johri, Ph.D. thesis, p 9[5])

> "Transfers of mycelium of the fungus *Cyathus helenae* were grown throughout as 'Still-Surface' cultures. Brodie medium, without agar and containing 2% dextrose (but no maltose), was used during the earlier part of the work. A quantity of 200 ml or 400 ml of this medium was dispensed into a 500 ml or 1,000 ml Erlenmeyer flask and autoclaved. The medium was inoculated with three or four 8.0 mm discs of mycelium of *Cyathus helenae* taken from a 16 to 18-day old colony growing on Brodie solid medium. During the summer of 1968, as much as 45 liters of broth were extracted. The mycelium was removed by passing the broth from cultures (25-day-old) through a thick layer of cheese cloth. The broth was then extracted using an equal volume of ethyl acetate. The yellowish ethyl acetate extract, along with some aqueous dark-colored impurities, was again passed through a thick layer of cheese cloth. The resulting yellow liquid was reduced to dryness under vacuum at 35°C. This operation produced a reddish-brown powder, highly soluble in acetone or ethyl acetate."

See Introduction for the composition of Brodie medium. From 1 l. was obtained 0.2 - 1.0 g crude cyathin.

Isolation of Chromocyathin

Crude cyathin complex (1.7 g; part of October,

1969 crop) was triturated with chloroform (15 ml). The undissolved material (0.59 g) was obtained as a microcrystalline yellowish-brown solid after filtration. Sublimation (140-170°/0.3 mm) followed by recrystallization from chloroform-methanol (9:1) gave yellow crystals of chromocyathin, mp 216-219° (dec); uv (MeOH): λ_{max} 241 (ε 14,000), 282 (ε 11,000) 350 nm (ε 8,000); ir (nujol): 3380, 3160, 1630, 1610, 1590 cm^{-1}; nmr (acetone-d_6): δ 9.75 (1H, s, C$\underline{H}$O), 7.13 (1H, s, aromatic H), 6.43 (1H, s, aromatic H); mass spectrometry: calcd mw for $C_7H_6O_4$: 154, found: 154; hrms of m/e 153, calcd for $C_7H_5O_4$: 153.0188, found: 153.0187.

Synthesis of 2,4,5-Trihydroxybenzaldehyde

1) 1,2,4-Triacetoxybenzene[13, 14]

A mixture of benzoquinone (48 g, 0.44 mole), acetic anhydride (144 g, 1.41 mole) and conc H_2SO_4 (5 ml) was kept at 50° for 2 hr. The resulting paste was suspended in water and the precipitate was collected by filtration. The filter cake was recrystallized from 50% ethanol (200 ml). Yield ∿100 g (90%). Characterized by nmr only ($CDCl_3$): δ 7.1 (3H, m, 3 aromatic H), 2.23 (9H, s, 3 x $O_2C\underline{CH_3}$).

2) 1,2,4-Trihydroxybenzene[12, 13]

A mixture of 1,2,4-triacetoxybenzene (100 g),

methanol (200 ml) and conc H_2SO_4 (10 ml) was heated under reflux for 1 hr. The reaction mixture was neutralized with Na_2CO_3 (20 g), taken up in ether and washed with saturated NaCl solution. The ethereal phase was dried with $CaCl_2$ and the ether was evaporated under reduced pressure. The residue solidified on standing in an evacuated desiccator, mp ∿130°. The yield was not determined. Characterized by nmr only (acetone-d_6): δ 7.5 (2H, broad, 2 x O$\underline{H}$), 6.65 (1H, d, J = 8 Hz, aromatic H), 6.40 (1H, d, J = 3 Hz, aromatic H), 6.18 (1H, dd, J = 8, 3 Hz, aromatic H), 3.4 (1H, broad, O$\underline{H}$).

3) 2,4,5-Trihydroxybenzaldehyde: Cyanide Method[12,15,16]

1,2,4-Trihydroxybenzene (6.3 g, 0.05 mole) was dissolved in ether (100 ml) and potassium chloride (0.1 g) and zinc cyanide (11.7 g, 0.1 mole) added. The suspension was cooled in ice and vigorously stirred while a rapid stream of dry hydrogen chloride was passed through the mixture for 1 hr. The ether was then decanted and ice (50 g) was added to the residue. The resulting mixture was heated on the steam bath and the reddish precipitate (3.0 g, 39%) removed by filtration. Sublimation of a portion of this material followed by crystallization from chloroform-methanol gave 2,4,5-trihydroxybenzaldehyde, mp 216-219° (dec), reported 223°[12]. This material had the same spectral characteristics as chromocyathin (natu-

ral material).

4) 2,4,5-Trihydroxybenzaldehyde: Orthoformate Method[17]

1,2,4-Trihydroxybenzene (12.5 g, 0.1 mole), triethyl orthoformate (100 ml, excess) and ether (300 ml) were placed in a 1-liter three-necked flask fitted with condenser and mechanical stirrer. The mixture was cooled in ice and aluminum chloride (10 g, 0.15 mole) was added over a period of 5 min. The mixture was stirred at rt for 0.5 hr and subsequently poured into 5% hydrochloric acid (500 ml). The product was extracted with ether (a total of 1 l.), the extract washed with $NaHCO_3$ solution until neutral, dried ($MgSO_4$) and the ether evaporated under reduced pressure. Recrystallization of the residue from chloroform-methanol gave brown crystals of slightly impure 2,4,5-trihydroxybenzaldehyde (6.9 g, 45%). The low yields are likely due to the fact that the product decomposes, particularly when exposed to air in alkaline solution. In order to remove the brown impurities, the material was sublimed (150-170°/0.1 mm). The spectra were identical with those of the other preparations. A microanalysis was also performed. Anal. calcd for $C_7H_6O_4$: C, 54.55; H, 3.92; found: C, 54.35; H, 4.17.

Preparation of 2,4,5-Triacetoxybenzaldehyde

2,4,5-Trihydroxybenzaldehyde (1.15 g, 0.0075

mole) was dissolved in pyridine (12 ml) and cooled in ice. Acetic anhydride (6 ml, 0.06 mole) was added gradually. The mixture was allowed to stand at rt for 10 min, then poured into cold water (100 ml), stirred and filtered. Recrystallization from 98% ethanol yielded 2,4,5-triacetoxybenzaldehyde (1.139 g, 58%), mp 114-115°, reported 115°[18]; ir ($CHCl_3$): no hydroxyl, 1760, 1690 cm^{-1}; nmr ($CDCl_3$): δ 10.13 (1H, s, $C\underline{H}O$), 7.72 (1H, s, aromatic H), 7.20 (1H, s, aromatic H), 2.35 (3H, s, $O_2CC\underline{H_3}$), 2.28 (6H, s, 2 x $O_2CC\underline{H_3}$); hrms: calcd for $C_{13}H_{12}O_7$: 280.0583; found: 280.0584; mass spectrum: m/e 280 (0.3), 238 (10), 196 (60), 154 (100), 153 (10). Anal. calcd for $C_{13}H_{12}O_7$: C, 55.72; H, 4.32; found: C, 55.98; H, 4.21.

On standing in ethanol the material reverts to 2,4,5-trihydroxybenzaldehyde and decomposes further.

Chromocyathin ("natural material", 0.23 g) was treated in an analogous way to afford the acetyl derivative (0.11 g), mp 114-115°, undepressed on admixture of "synthetic" 2,4,5-triacetoxybenzaldehyde. Ir and nmr spectra were identical with those of synthetic material.

Initial Separation of Crude Cyathin Extract: Column Chromatography

1) General Procedure:

After stirring the crude extract with a small volume of chloroform (1 ml per 100 mg), remove insoluble

chromocyathin ($C_7H_6O_4$) by filtration.

Subject the filtrate (after evaporation) to column chromatography on silicic acid (Mallinckrodt). The ratio of adsorbent to substrate should be 40:1. Select a column in such a way that the adsorbent bed has a height of 100 mm.

Use three solvent systems as eluents:

F) chloroform

G) chloroform-methanol (98:2)

H) chloroform-methanol (95:5)

System F will elute group I and group II; system G, group III; and system H, group IV and group V constituents. (See Chapter II, p 16, for the group classifications.) Monitor the eluate frequently by tlc in order to change the solvent systems at the right time and to assign the various fractions to groups I-V.

2) Example:

On January 7, 1970, 2.82 g chromocyathin-free extract (a portion of the material obtained from the October '69 growth) was chromatographed on 82 g of silicic acid. The adsorbent column had a height of 90 and a diameter of 55 mm. The substrate was eluted with 2.2 l. of system F (group I and II eluted), 1.1 l of G (group II, III, and IV) and 1.0 l of H (group IV and V).

After evaporation of the solvent, 0.086 g group I,

0.105 g group II, 0.385 g group III, 0.176 g group IV, and 0.683 g group V components were obtained.

In group I, cyathin B_3, (R_f (B) 0.6; R_f (H) 0.6) was the major component. It was further purified by column chromatography on silicic acid and by sublimation (100-110°/0.05 mm); hrms: $C_{20}H_{28}O_3$ (calcd 316.2039, found 316.2038). Mass and ir spectra of cyathin B_3 are reproduced in Chapter II.

Group II contained at least two components. One of the fractions partially crystallized. A few of the crystals (allocyathin A_4) were collected, mp 85-90°, R_f (B) 0.5, hrms: $C_{20}H_{30}O_4$ (calcd: 334.2144; found: 334.2139). Mass and ir spectra of allocyathin A_4 are reproduced in Chapter II.

Group III contained chromocyathin, as indicated by the yellow color of the solution and tlc characteristics, R_f (B) 0.43, and at least two other components: one of these was cyathin A_3, R_f (B) 0.31, and the other possibly cyathin B_4, R_f (B) 0.39.

Group IV contained at least two components, as yet unidentified, R_f (B) 0.18 and 0.15.

The major component in group V was cyathin A_4, R_f (B) 0.09, hrms: $C_{20}H_{30}O_4$ (calcd 334.2144; found 334.2146). Mass and ir spectra of cyathin A_4 are reproduced in Chapter II.

Initial Separation of Crude Cyathin Extract: Thin-Layer Chromatography

Two examples will be given to illustrate the method.

1) Separation of April '69 crop in May '69

This batch had been produced by using a culture medium that contained maltose (60 g/l.) as the sugar source. It is known as batch VI. The broth (4 l.) was extracted with ethyl acetate (4 l.). After drying and evaporation, 3.03 g of crude extract was obtained. Extraction with 5%-$NaHCO_3$ solution removed some of the chromocyathin. When the residue from this process was stirred in ether, more chromocyathin could be removed by filtration. The residue (2.01 g) now contained only minor amounts of chromocyathin, as judged by tlc.

This material was applied manually to six 20 x 100 cm tlc plates (silica gel G containing electronic phosphor, 0.5 mm thick). Solvent system C was used for developing. Several bands were detected under short-wave uv-light. The layers were divided into five zones, scraped off, and the organic material was eluted from the adsorbent with acetone.

Group	R_f (C) range	amount [mg]
I	0.7 - 0.8	68
II	0.6 - 0.7	133

Group	R_f (C) range	amount [mg]
III	0.4 - 0.6	583
IV	0.15- 0.4	383
V	0.05- 0.15	373

The major component in group V was cyathin A_4 (tlc).

Group IV contained a mixture (tlc) and was not further investigated.

Group II contained several unidentified components.

The major component of Group I was cyathin B_3, identical with the component mentioned above (tlc).

Group III contained chromocyathin which was removed by stirring with chloroform and subsequent filtration. No cyathin A_3 was found in the filtrate, but a new compound, cyathin B_4, R_f (C) 0.5, was detected. This material was further purified by ptlc. Attempts at crystallization and sublimation were unsuccessful. The molecular formula was determined by hrms: $C_{20}H_{28}O_4$ (calcd 332.1988, found 332.1985). Mass and ir spectra of cyathin B_4 are reproduced in Chapter II.

The preparation of derivatives of cyathin B_4 such as the acetate and acetonide was unsuccessful.

2) Separation of July '69 crop in August '69

This batch was considered somewhat irregular since part of the fungal mycelium had submerged during

cultivation.

After extraction with 11 l. of ethyl acetate, 11 l. of broth yielded 2.71 g of crude extract; 2.31 g of the crude extract was ether soluble.

The ether soluble extract (1.96 g) was subjected to ptlc on five 20 x 100 cm plates, as described above. After developing and eluting, as also described above, the following fractions were obtained: combined groups I and II, 384 mg; III, 620 mg; IV, 270 mg; and V, 504 mg.

According to tlc, all groups consisted of several components, except possibly group V, in which cyathin A_4 appeared to predominate. Combined groups I and group II probably contained some cyathin B_3, but were not investigated further at this stage. Constituents of group IV were not investigated.

Group III again contained chromocyathin which was removed by stirring with chloroform and filtration. The filtrate, which contained cyathin A_3 as a major component (tlc), was further purified by acid-base separation. (It was found later that cyathin A_3 deteriorates if allowed to stand in alkaline solution). In one instance, 465 mg of the group III constituents gave 373 mg chloroform soluble; 102 mg chloroform soluble gave 41 mg neutral material. This isolation procedure was probably quite wasteful. Material thus obtained appeared rather pure, R_f (C) 0.36, hrms: $C_{20}H_{30}O_3$. The last purification step

was sublimation (170°/0.05 mm); the "sublimate" was a colorless glass that "melted" at 40-45°. At the time, this was considered very pure cyathin A_3, but actually proved to be what is called "A_3 mixture" in Chapter II. Spectra were recorded and derivatives prepared; however, they will not be discussed here, since this material was a mixture of what is now known as cyathin A_3 and allocyathin B_3.

Preparation of Crude Cyathin by Ayerst Laboratories and Isolation of Patulin

Report received from Ayerst Laboratories (March 1971):

> "120 Fernbach flasks containing 300 ml of the medium were inoculated with 8 mm discs of 16 days' growth on agar of *Cyathus Helenae* F-638. The flasks were incubated at 22°C for 29 days. At the end of incubation period, 32 litres of the filtrate were recovered by filtration. The filtrate was extracted twice with one v/v of ethyl acetate, the ethyl acetate extracts were combined, dehydrated over sodium sulfate and evaporated to dryness to yield about 12 grams of dry residue. This residue has m.i.c. against *Staph. aureus* of 6.5 μg/ml."

Patulin was found in both of the two Ayerst preparations of crude cyathin. It was a group III constituent and was separated by column chromatography followed by thin-layer chromatography according to the general methods

outlined on the preceeding pages. Its tlc characteristics (high sensitivity to the uv-detection method, but insensitivity to the H_2SO_4-detection method) distinguished it from the terpenoid cyathin components. The crude patulin was not purified to the extent that it would crystallize. However, the spectral data identify the material as patulin[53]; hrms: calcd for $C_7H_6O_4$: 154.0264, found: 154.0265; ir ($CHCl_3$): 3680 (weak), 3580 (sharp), 3500 (broad), 2950, 2860 (weak C-H), 1780 (very strong), 1750, 1675, 1595 cm^{-1}; nmr ($CDCl_3$): δ 5.8-6.1 (s(1 H) + m(2 H)), 4.64 (1 H, dd, J = 17.3), 4.35 (1 H, dd, J = 17.4); uv (MeOH): ε_{276} 12,500 (max); nmr of O-acetylpatulin (obtained by following acetylation procedure 3), but substituting benzene for methylene chloride) (CCl_4): δ 6.90 (1 H, s), 6.01 (1 H, dd, J = 2.5, 1.5), 5.90 (1 H, ddd, J = 4.5, 2.5, 2.5), 4.68 (1 H, ddd, J = 17.5, 2.5, 1.5), 4.43 (1 H, dd, J = 17.5, 4.5), 2.07 (3 H, s, $OCOC\underline{H_3}$).

Separation of "A_3-Mixture" by Chromatography on Silver Nitrate-Silica Gel

1) Column Chromatography

The adsorbent, 10% silver nitrate-silica gel, was prepared by dry mixing of 25% silver nitrate-silica gel (Adsorbosil, 60/100 mesh, Applied Science Laboratories, Inc.) and ordinary silica gel (Fisher, 28/200 mesh, grade

12) in the ratio 2:3 with the aid of a rotary evaporator.

Chromatographically pure (by ordinary tlc) "A_3-mixture" (266 mg) was chromatographed on 20 g of 10% silver nitrate-silica gel using a 100-ml buret as a column (adsorbent bed 16 cm high). 600 ml of solvent system K and 300 ml of L were used as eluents.

The first 150 ml eluate contained no material. The next 300 ml contained 95 mg of rather pure cyathin A_3, R_f ($AgNO_3$, N) 0.60. One of the 50-ml fractions crystallized spontaneously on evaporation of the solvent, and two others were induced to crystallize. The remaining 450 ml contained mixtures (altogether 58 mg) of cyathin A_3 and allocyathin B_3, R_f ($AgNO_3$, N) 0.51.

This experiment was carried out six times: in none of the cases could pure allocyathin B_3 be obtained. Nmr spectroscopy provided useful criteria to estimate the composition of the mixtures: protons a of the two cyathins appear at slightly different chemical shifts (δ 5.99 in cyathin A_3 and δ 6.02 in allocyathin B_3). The ratio of the integrated signals reflects the ratio of the two components.

2) Thin-Layer Chromatography

a) General Procedure

Apply approximately 120 mg "A_3-mixture" (pure

according to ordinary tlc) to a 0.05 x 20 x 100 cm thin-layer of 10% silver nitrate-silica gel. Develop in solvent system N until the solvent front has almost reached the top of the plate. Allow for a short drying period under atmospheric conditions and spray the plate very sparsely with a 0.2% solution of 2',7'-dichlorofluorescein in ethanol. Locate the separated cyathin A_3 and allocyathin B_3 bands at R_f 0.5-0.7 by viewing under long-wave uv-light (325 nm), mark and scrape off. Place each scraping in a chromatographic column of such dimensions that an adsorbent layer of ca. 10 cm results and elute each slowly (1 drop/sec) with 150 ml ether. Evaporate the ether and obtain cyathin A_3 and allocyathin B_3. Upon trituration with acetone, cyathin A_3 should crystallize. Further purification steps are necessary to obtain crystalline allocyathin B_3.

b) Example

The above procedure was followed on March 23, 1971. Ca. 120 mg "A_3-mixture" was applied to each of two plates. After development and detection, the cyathin A_3 and allocyathin B_3 fractions were combined; each fraction was eluted with 250 ml ether. After evaporation, 117 mg cyathin A_3 and 79 mg allocyathin B_3 were obtained. Cyathin A_3 crystallized after the addition of a few drops of

acetone.

Crystallization and Characterization of Cyathin A_3

Cyathin A_3 (purified by ptlc on $AgNO_3$-silica gel, partially crystalline, approximately 15 mg) was placed in a 2 ml Erlenmeyer flask and dissolved in hot benzene (∿0.5 ml). Cyclohexane was added dropwise until the solution became cloudy. A few drops of benzene were added to dissolve the precipitate. The solution was then allowed to cool to room temperature and to stand undisturbed overnight. When observed the next morning, crystals had appeared, but they were too small for X-ray crystallographic studies. The solution was warmed until the crystals dissolved, a few more drops of benzene were added and the solution was allowed to stand for a day. Again, the crystals were too small and the procedure was repeated several times. After a week's experimentation a concentration was obtained which allowed sufficiently slow crystal growth. The largest crystals thus obtained were of the size 1.0 x 0.3 x 0.3 mm; mp 148-150°; hrms: calcd for $C_{20}H_{30}O_3$: 318.2195, found: 318.2195; $[\alpha]_D$ -155° (C, 0.26, methanol). Mass, ir, nmr, uv, ord and cd spectra are reproduced in Chapter II.

Crystallization and Characterization of Allocyathin B_3

All analogous crystallization attempts with allocyathin B_3 (purified by ptlc on $AgNO_3$-silica gel, noncrystalline) were unsuccessful.

Crystals of allocyathin B_3, however, were obtained accidentally in the unplanned methanolysis experiment mentioned below.

Other preparations of allocyathin B_3 could now be crystallized from benzene-Skellysolve B by using these crystals as seeds. Still, crystallization did not proceed as smoothly as in the case of cyathin A_3: the solutions had to be fairly concentrated before crystallization occurred and the resulting crystals were always microscopically small; mp 143-144°, hrms: calcd for $C_{20}H_{28}O_3$: 316.2039, found: 316.2031. The mass and ir spectra are reproduced in Chapter II. The following characteristic data were obtained from noncrystalline allocyathin B_3: $[\alpha]_D$ -250° (C, 0.26, methanol); nmr ($CDCl_3$): δ 6.30 and 6.23 (2H, AB quartet, J = 5 Hz, C$\underline{H}$ = C$\underline{H}$), 6.02 (1H, m, C = C$\underline{H}$), remainder of spectrum ill-defined; uv (methanol): λ_{max} 235 nm (ε 5,100); ord (C, 0.26, methanol): $[\phi]_{300}$ -16,000°, $[\phi]_{270}$ -24,000° (trough); (C, 0.07): $[\phi]_{256}$ -15,000° (peak), $[\phi]_{248}$ -15,000 (trough), $[\phi]_{223}$ +2,400 (peak); cd (C, 0.26, methanol): $\Delta\varepsilon_{327}$ +1.6 (pos max).

Acetylation of Cyathin A_3

Crystalline cyathin A_3 (20 mg, 0.063 mmole) was subjected to acetylation procedure 3). Monoacetylation required 30 min at 26°; diacetylation, 10 hr at 35°; monoacetate: R_f (M) 0.3; diacetate: R_f (M) 0.4, R_f ($AgNO_3$, M) 0.4. Work-up after 10 hr yielded crude O,O-diacetylcyathin A_3 (20 mg), a nearly colorless gum containing traces of impurity. The material was subjected to ptlc (19 mg) and sublimation at 105-120°/0.02-0.2 mm (15 mg recovered). The fingerprint region of the ir spectrum remained poorly defined and it is doubtful whether much further purification was effected. Characteristic data: hrms: calcd for $C_{24}H_{34}O_5$: 402.2406, found: 402.2404; $[\alpha]_D$ -64° (C, 0.13, isooctane); uv (isooctane): ε_{230} 7,300 (sh), ε_{289} 73 (min), ε_{323} 126 (max); ord (C, 0.13, isooctane): $[\phi]_{538}$ -280° (trough), $[\phi]_{363}$ +2,700° (peak), $[\phi]_{300}$ -12,300 (inflection), $[\phi]_{261}$ -22,500 (trough), $[\phi]_{235}$ -13,200 (peak); cd (C, 0.13, isooctane): $\Delta\varepsilon_{330}$ +2.73 (pos max), $\Delta\varepsilon_{244}$ -3.3 (neg min). Mass, ir and nmr spectra are reproduced in Chapter II.

Hydrolysis of O,O-Diacetylcyathin A_3

1) O,O-Diacetylcyathin A_3 (23 mg, contaminated with O,O-diacetylallocyathin B_3) was treated under nitrogen with 2% potassium carbonate in 80% aqueous methanol (1.5 ml) for 0.5 hr. The reaction mixture was diluted with

water (2 ml), extracted with ether (25 ml) and washed with water until neutral. Evaporation of the dried ($MgSO_4$) ether extract yielded crude product (16 mg), which was identical with cyathin A_3 (contaminated with allocyathin B_3) according to tlc, mass and ir spectra.

2) O,O-Diacetylcyathin A_3 (14 mg, uncontaminated by O,O-diacetylallocyathin B_3) was allowed to stand in methanol (10 ml) for a week. Evaporation yielded a residue that lacked ir absorption at 1745 cm^{-1} and whose tlc characteristics were similar to those of cyathin A_3. Trituration with acetone yielded crystals whose ir spectrum was identical with that of authentic cyathin A_3.

Ketalization of Cyathin A_3

Crystalline cyathin A_3 (20 mg), dissolved in methanol (10 ml), was treated with a saturated solution of hydrogen chloride in methanol (10 drops) for 0.5 hr. The mixture was then neutralized with solid sodium carbonate (120 mg). The methanol was evaporated, the residue taken up in ether, the suspension filtered and the ether evaporated to yield cyathin A_3 methyl ketal (22 mg), R_f (M) 0.29. This material was used to obtain spectral data without further purification; hrms: calcd for $C_{21}H_{32}O_3$: 332.2352, found: 332.2356; $[\alpha]_D$ -154° (C, 0.24, methanol); uv (methanol): end absorption only; ord (C, 0.24,

methanol): $[\phi]_{589}$ -510°, $[\phi]_{400}$ -1,290°, $[\phi]_{250}$ -7,900°; cd (C, 0.24, methanol): $\Delta\varepsilon_{325}$ 0.0. Mass, ir and nmr spectra are reproduced in Chapter II.

Hydrolysis of Cyathin A_3 Methyl Ketal

Cyathin A_3 methyl ketal (40 mg, obtained from impure cyathin A_3, but itself pure according to nmr and tlc) was allowed to stand in acetone-water-conc hydrochloric acid (20:2:1) for 18 hr at 32°. The mixture was neutralized with solid sodium carbonate, filtered and most of the acetone evaporated under reduced pressure. The resulting aqueous suspension was extracted with ether, dried ($MgSO_4$), filtered and evaporated to give crude product (36 mg). Purification by ptlc (silica gel, 0.05 x 20 x 20 cm, solvent system I) gave cyathin A_3 (11 mg) which crystallized upon trituration with acetone. Identity was established by tlc and ir spectrum (KBr-pellet).

Acetylation of Cyathin A_3 Methyl Ketal

Cyathin A_3 methyl ketal (20 mg, obtained from crystalline cyathin A_3) was subjected to acetylation procedure 3) (1.25 hr at 26° and 1 hr at 40°). Work-up yielded non-crystalline O-acetylcyathin A_3 methyl ketal (20 mg), R_f (M) 0.57, which contained traces of impurities (tlc). This material was used for spectral characteriza-

tion without further purification; hrms: calcd for $C_{23}H_{34}O_4$: 374.2457, found: 374.2450; ir (CCl_4): no hydroxyl, 1740 cm^{-1} (ester carbonyl); uv (isooctane): end absorption only; ord (C, 0.18, isooctane): $[\phi]_{300}$ -1,250°, $[\phi]_{250}$ -4,800°, $[\phi]_{230}$ -11,900° (trough); cd (C, 0.18, isooctane): $\Delta\varepsilon_{325}$ 0.0. The mass spectrum is reproduced in Chapter II; the nmr data are listed in Table III.

Acetylation of Allocyathin B_3

In two experiments, noncrystalline allocyathin B_3 (50 mg) was subjected to acetylation procedure 2). The crude product (51 mg) was purified by ptlc on 10% $AgNO_3$-silica gel (developed with system M) to give O,O-diacetylallocyathin B_3 (24 mg), R_f (M) 0.4, R_f ($AgNO_3$, M) 0.4, which could not be crystallized; uv (methanol): ε_{215} 14,000 (min), ε_{225} 16,000 (max); ε_{250} 9,700 (inflection), ε_{300} 260; ord (C, 0.26, methanol): $[\phi]_{365}$ -1,200° (peak); (C, 0.13): $[\phi]_{320}$ -9,200° (inflection), $[\phi]_{300}$ -15,000° (inflection), $[\phi]_{272}$ -22,000° (trough), $[\phi]_{250}$ -18,000° (shoulder), $[\phi]_{222}$ +2,500° (peak); cd (C, 0.26 methanol) $\Delta\varepsilon_{325}$ +1.6 (pos max). (These data should be considered with caution, since the sample had undergone methanolysis to an unknown extent.) Mass and ir spectra were not secured; the nmr spectrum is reproduced in Chapter II.

Methanolysis of O,O-Diacetylallocyathin B_3

O,O-Diacetylallocyathin B_3 (13 mg, purified at this stage by ptlc on $AgNO_3$-silica gel) was allowed to stand in methanol (10 ml) for a week. Evaporation yielded a residue that lacked ir absorption at 1745 cm^{-1} and whose tlc characteristics were similar to those of allocyathin B_3.

After storage for five months in the refrigerator, the residue had partially crystallized.

Ketalization of Allocyathin B_3

Noncrystalline allocyathin B_3 (31 mg), dissolved in methanol (20 ml), was treated with a saturated solution of hydrogen chloride in methanol (1 ml) for 0.5 hr. Freshly ground sodium carbonate (0.8 g) was added and stirred until the solution was neutral. Undissolved material was filtered off and the solvent removed by evaporation under reduced pressure. The residue was taken up in ether, filtered and evaporated to give crude allocyathin B_3 methyl ketal (28 mg), which was purified by ptlc (developed with system M). The purified material (20 mg), R_f (M) 0.39, could not be crystallized; hrms: calcd for $C_{21}H_{30}O_3$: 330.2195, found: 330.2190; $[\alpha]_D$ - 230° (C, 0.18, isooctane); uv (isooctane): ε_{228} 1,900 (min), ε_{256} 4,200 (max),

ε_{320} 0; ord (C, 0.18, isooctane): $[\phi]_{589}$ -750°, $[\phi]_{400}$ -2,100°; (C, 0.07): $[\phi]_{300}$ - 6,200°, $[\phi]_{273}$ -14,400° (trough), $[\phi]_{256}$ -7,300° (inflection), $[\phi]_{240}$ -180° (peak); cd (C, 0.07, isooctane): $\Delta\varepsilon_{252}$ -3.6 (neg min). Mass, ir and nmr spectra are reproduced in Chapter II.

Acetylation of Allocyathin B_3 Methyl Ketal

Impure cyathin B_3 methyl ketal (20 mg; not the material mentioned above) was subjected to acetylation procedure 3) (1 hr at 30°, 0.5 hr at 40°). Acetylation was essentially complete after 1 hr (tlc). The crude product (18 mg) was purified by ptlc (developed with system M) to give O-acetylallocyathin B_3 methyl ketal (6 mg, yield much reduced due to spillage), R_f (M) 0.55, which could not be crystallized; hrms: calcd for $C_{23}H_{32}O_4$: 372.2301, found: 372.2305; $[\alpha]_D$ -230° (C, 0.1, isooctane); ir (CCl_4): no hydroxyl, 1740 cm^{-1} (ester carbonyl); uv (isooctane): ε_{229} 2,900 (min), ε_{255} 5,600 (max); ord (C, 0.1, isooctane): $[\phi]_{589}$ -750°, $[\phi]_{400}$ -1,400°, $[\phi]_{300}$ -4,600°, $[\phi]_{273}$ -10,000 (trough), $[\phi]_{250}$ -4,600° (inflection), $[\phi]_{233}$ -90° (peak); cd (C, 0.1, isooctane): $\Delta\varepsilon_{243}$ -2.2 (neg min). The mass spectrum is reproduced in Chapter II; the nmr data are listed in Table V.

Hydrogenation of Cyathin A_3 Methyl Ketal

Crystalline cyathin A_3 (30 mg), dissolved in

methanol (60 ml), was treated with a saturated solution of hydrogen chloride in methanol (1 ml) for 0.5 hr. Dry, freshly ground sodium carbonate (1 g) was added, and the mixture stirred until the solution was neutral, and then the insoluble material was filtered off. Palladium-charcoal (5%, 50 mg) was added to the filtrate and the mixture stirred in an atmosphere of hydrogen for 0.5 hr at room temperature. The catalyst was removed by filtration and the solvent evaporated under reduced pressure. The residue was taken up in ether, filtered, and the solvent evaporated under reduced pressure. The crude product (28 mg) consisted mainly of two components, alcohol **47**, R_f (M) 0.37, and aldehyde **50**, R_f (M) 0.55, along with trace amounts of **48**, R_f (M) 0.70 (identified by tlc). The aldehyde (12 mg) and the alcohol (7 mg) were separated by ptlc (developed with system M). No attempt was made to isolate **48**.

Alcohol **47** failed to crystallize; hrms: calcd for $C_{21}H_{34}O_3$: 334.2508, found: 334.2503; ir (CCl_4): 3630 cm^{-1} (hydroxyl); mass spectrum: m/e 334 (2.1), 319 (2.6), 190 (100), 175 (40), 147 (7.0). (Peaks at m/e 220 and 205 are omitted as they are attributed to a frequently encountered impurity).

Aldehyde **50** crystallized spontaneously after evaporation of the solvent from the appropriate chromatographic fraction and was recrystallized from Skellysolve B.

The crystals formed a slush at 113-115°C, which did not give a transparent melt even at 135°; hrms: calcd for $C_{21}H_{32}O_3$: 332.2352, found: 332.2343; $[\alpha]_D$ -39° (C, 0.22, isooctane); uv (isooctane): ε_{275} 124 (max), ε_{282} 122 (max), 4 shoulders between 290-340 nm; ord (C, 0.22, isooctane): $[\phi]_{700}$ -72°, $[\phi]_{589}$ -96°, $[\phi]_{340}$ -126° (trough), $[\phi]_{323}$ +1,370° (peak), $[\phi]_{318}$ +1,240° (trough), $[\phi]_{312}$ +1,600 (peak), $[\phi]_{270}$ -4,200° (trough); cd (C, 0.22, isooctane): $\Delta\varepsilon_{290}$ +1.2 (pos max with fine structure). Mass, ir and nmr spectra are reproduced in Chapter II.

Hydrogenation of Allocyathin B_3 Methyl Ketal

Allocyathin B_3 methyl ketal (purified by ptlc, 10 mg), dissolved in methanol (20 ml), was hydrogenated over palladium-charcoal (5%, 20 mg) at atmospheric pressure and room temperature for 17 min. The catalyst was filtered off and the solvent evaporated under reduced pressure. The crude product (9 mg) consisted of three components, R_f (M) 0.34, 0.51 and 0.68. The components were separated by ptlc (developed with system M) and were found (see below) to be 47 (1 mg), 49 (1 mg) and 48 (3 mg), respectively.

Compound 47 had the same ir spectrum and R_f-value as the one mentioned above. The mass spectrum differed only slightly: m/e 334 (2.6), 3.19 (2.6), 190 (100),

174 (42), 147 (8.2).

Compound 48 had the same tlc characteristics as the one mentioned above; calcd for $C_{21}H_{34}O_2$: 318.2559, found: 318.2550; ir (CCl_4): no hydroxyl, no carbonyl absorption; mass spectrum: 318 (3.1), 316 (2.1), 303 (3.1), 190 (100), 175 (43), 147 (11.9).

Compound 49, whose ir spectrum displayed aldehyde absorption, was slightly more polar than aldehyde 50 (R_f (M) 0.51 vs. 0.54). Aldehyde 49 was converted to 50 by being allowed to stand in a saturated solution of sodium carbonate in methanol (1 ml) for 28 hr at room temperature. The solvent was then evaporated under reduced pressure, the residue taken up in ether, filtered and evaporated under reduced pressure. The ir spectrum and tlc characteristics of the residue were indistinguishable from those of aldehyde 50 obtained from cyathin A_3. The small amount (∿0.5 mg), however, precluded attempts at crystallization.

The different product distribution has been attributed to the fact that the hydrogenations were carried out at different pH values: The medium was slightly alkaline in the case of cyathin A_3 and neutral in the case of allocyathin B_3 methyl ketal.

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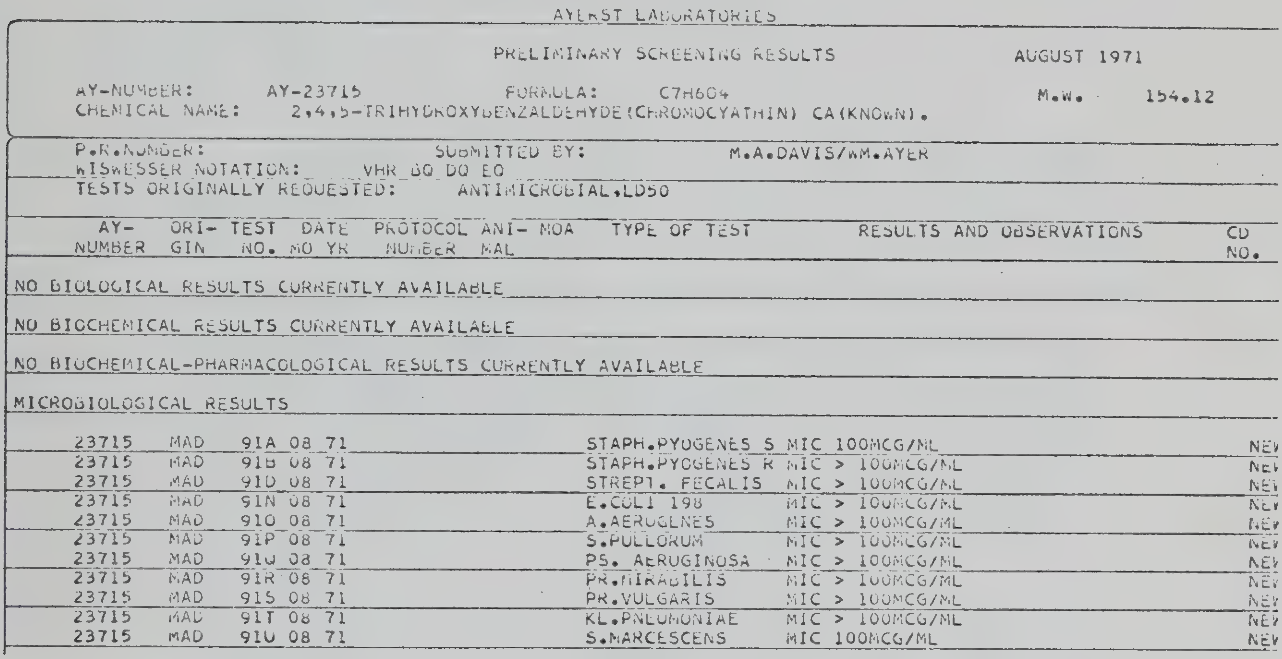
APPENDIX 1

Results of Microbiological Tests of Chromocyathin and "A_3-Mixture"



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                                    AYERST LABORATORIES

                              PRELIMINARY SCREENING RESULTS              AUGUST 1971

   AY-NUMBER:       AY-23715         FORMULA:      C7H6O4                 M.W.      154.12
   CHEMICAL NAME:     2,4,5-TRIHYDROXYBENZALDEHYDE(CHROMOCYATHIN) CA(KNOWN).

   P.R.NUMBER:                     SUBMITTED BY:                M.A.DAVIS/WM.AYER
   WISWESSER NOTATION:      VHR BQ DQ EQ
   TESTS ORIGINALLY REQUESTED:      ANTIMICROBIAL,LD50

     AY-    ORI- TEST  DATE  PROTOCOL ANI- MOA   TYPE OF TEST         RESULTS AND OBSERVATIONS     CD
   NUMBER   GIN   NO. MO YR   NUMBER  MAL                                                          NO.

 NO BIOLOGICAL RESULTS CURRENTLY AVAILABLE

 NO BIOCHEMICAL RESULTS CURRENTLY AVAILABLE

 NO BIOCHEMICAL-PHARMACOLOGICAL RESULTS CURRENTLY AVAILABLE

 MICROBIOLOGICAL RESULTS

     23715    MAD    91A 08 71                     STAPH.PYOGENES S MIC 100MCG/ML                 NEW
     23715    MAD    91B 08 71                     STAPH.PYOGENES R MIC > 100MCG/ML               NEW
     23715    MAD    91D 08 71                     STREPT. FECALIS  MIC > 100MCG/ML               NEW
     23715    MAD    91N 08 71                     E.COLI 198       MIC > 100MCG/ML               NEW
     23715    MAD    91O 08 71                     A.AEROGENES      MIC > 100MCG/ML               NEW
     23715    MAD    91P 08 71                     S.PULLORUM       MIC > 100MCG/ML               NEW
     23715    MAD    91Q 08 71                     PS. AERUGINOSA   MIC > 100MCG/ML               NEW
     23715    MAD    91R 08 71                     PR.MIRABILIS     MIC > 100MCG/ML               NEW
     23715    MAD    91S 08 71                     PR.VULGARIS      MIC > 100MCG/ML               NEW
     23715    MAD    91T 08 71                     KL.PNEUMONIAE    MIC > 100MCG/ML               NEW
     23715    MAD    91U 08 71                     S.MARCESCENS     MIC 100MCG/ML                 NEW
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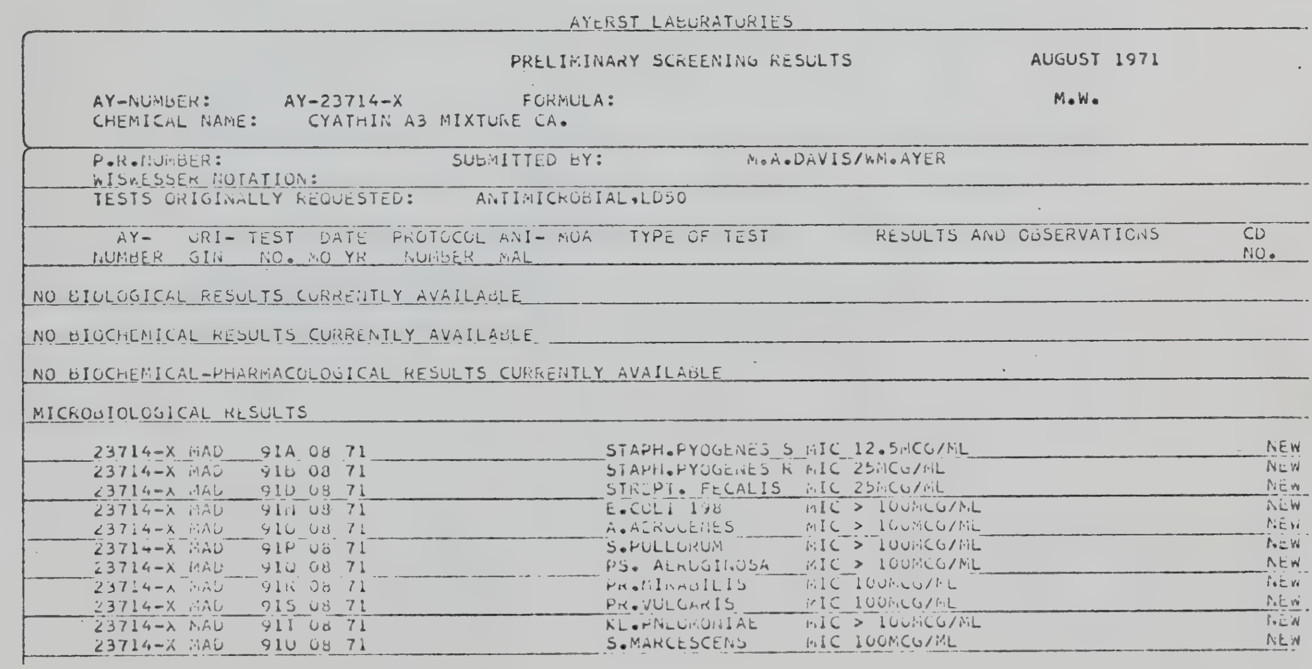

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                                    AYERST LABORATORIES

                              PRELIMINARY SCREENING RESULTS              AUGUST 1971

   AY-NUMBER:       AY-23714-X       FORMULA:                             M.W.
   CHEMICAL NAME:     CYATHIN A3 MIXTURE CA.

   P.R.NUMBER:                     SUBMITTED BY:                M.A.DAVIS/WM.AYER
   WISWESSER NOTATION:
   TESTS ORIGINALLY REQUESTED:      ANTIMICROBIAL,LD50

     AY-    ORI- TEST  DATE  PROTOCOL ANI- MOA   TYPE OF TEST         RESULTS AND OBSERVATIONS     CD
   NUMBER   GIN   NO. MO YR   NUMBER  MAL                                                          NO.

 NO BIOLOGICAL RESULTS CURRENTLY AVAILABLE

 NO BIOCHEMICAL RESULTS CURRENTLY AVAILABLE

 NO BIOCHEMICAL-PHARMACOLOGICAL RESULTS CURRENTLY AVAILABLE

 MICROBIOLOGICAL RESULTS

     23714-X MAD     91A 08 71                     STAPH.PYOGENES S MIC 12.5MCG/ML                NEW
     23714-X MAD     91B 08 71                     STAPH.PYOGENES R MIC 25MCG/ML                  NEW
     23714-X MAD     91D 08 71                     STREPT. FECALIS  MIC 25MCG/ML                  NEW
     23714-X MAD     91N 08 71                     E.COLI 198       MIC > 100MCG/ML               NEW
     23714-X MAD     91O 08 71                     A.AEROGENES      MIC > 100MCG/ML               NEW
     23714-X MAD     91P 08 71                     S.PULLORUM       MIC > 100MCG/ML               NEW
     23714-X MAD     91Q 08 71                     PS. AERUGINOSA   MIC > 100MCG/ML               NEW
     23714-X MAD     91R 08 71                     PR.MIRABILIS     MIC 100MCG/ML                 NEW
     23714-X MAD     91S 08 71                     PR.VULGARIS      MIC 100MCG/ML                 NEW
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APPENDIX 2

Crystal and Molecular Structure of Cyathin A_3

(Condensed version of a communication by M.J. Bennett and R.M. Tuggle[48]).

> "Cyathin A_3 crystallizes as colorless prisms in the orthorhombic space group $P2_12_12_1$, a = 18.228 (1), b = 15.105 (1), c = 6.629 (1) Å, z = 4. 1515 significant ($I/\sigma(i)>3$) independent intensities were measured on a Picker four circle diffractometer by the coupled $\omega/2\theta$ scan method using CuKα radiation. The structure was solved by direct methods and has been refined to a present conventional R factor of 0.037. All hydrogen atoms have been located.
>
> Solution of the structure has revealed that cyathin A_3 crystallizes in the hemiketal form. The relative structure and pertinent bond distances are given in the figure. A prominent structural feature of the molecule is that both six-membered rings assume chair conformations. Both five-membered rings show significant deviations from planarity. When bond shortening effects due to thermal motion are taken into consideration all bond distances within the molecule compare reasonably with expected values."

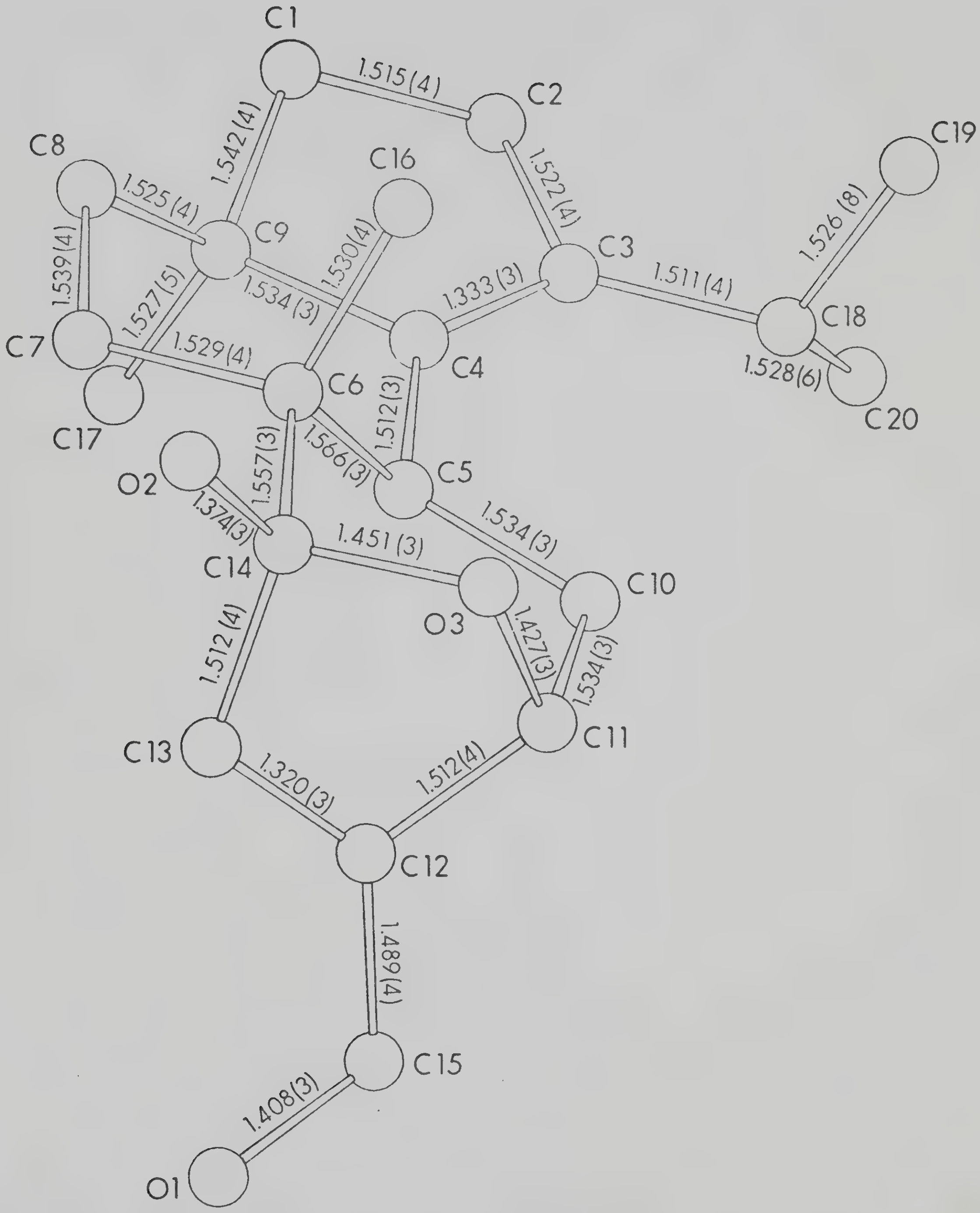

Figure. Molecular geometry of cyathin A_3.

Numbers in parentheses are the estimated standard deviations in the last figure quoted. Hydrogen atoms have been omitted for clarity.

B30044